ASPECTS OF THE SEROLOGICAL RELATIONSHIPS BETWEEN MEASLES, RINDERPEST AND CANINE DISTEMPER

VOLUME 1

by

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I declare that the work presented in this Thesis is my own.

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SUMMARY

The existence of distinct antigenic relationships between measles, rinderpest and canine distemper was confirmed and extended. The nature and extent of the serological cross-reactions were significantly influenced by the type of host and the degree of exposure to the viruses. In natural hosts, the homotypic and heterotypic antibody responses were similar. In heterologous hosts, however, exposures to the viruses resulted in antibody responses characterized by a range of cross-reactions which indicated that measles and rinderpest were more efficient antigens than canine distemper.

1. Measles virus was related to rinderpest virus through its envelope and nucleocapsid antigens such that measles haemagglutination and haemadsorption were specifically inhibited by measles and rinderpest antibodies. Moreover, measles virus reacted with rinderpest antibodies in complement-fixation and neutralization tests. However, no reaction occurred when measles virus was diffused through agar gels against rinderpest antibodies.

Measles virus was related to distemper virus through the nucleocapsid antigens such that measles antigens reacted with distemper antibodies in complement-fixation tests. In addition, a relationship through the envelope antigens was occasionally demonstrated; a few sera from distemper-convalescent dogs specifically inhibited measles haemagglutination. On the other hand, hyperimmune anti-distemper sera prepared in cattle, horses and rabbits never inhibited measles haemagglutination. Measles antibody neutralised distemper virus and reacted with distemper complement-fixing antigens but no link was evident in cross-immuno-diffusions tests.

Rinderpest and distemper viruses were related through the nucleocapsid antigens such that rinderpest antibodies neutralized distemper virus and reacted with distemper complement-fixation and precipitating antigens.

2. Single exposure of animals to measles resulted in the production of antibodies reactive with measles and distemper viruses. Anamnestic responses ensued in sensitized animals, on challenge with heterotypic live virus only.

Single inoculation of animals with rinderpest virus produced antibodies reactive with rinderpest, measles and distemper viruses.

Single exposure of dogs and multiple exposures of other animals to live distemper virus stimulated the production of distemper antibodies and sometimes antibodies reactive with measles antigens. Challenge inoculation with live or inactivated heterotypic virus resulted in anamnestic response.

3. The parameters of measles haemagglutination, haemadsorption, haemagglutination-inhibition, complement-fixation and neutralization tests were studied. Measles and distemper-infected tissues were rich in complement-fixation and precipitating antigens. Production of measles and distemper antigens in cell cultures, however, was poor and was not influenced by the cell type, the age of the cells,

dose of virus and temperature of incubation. The yield of measles haemagglutinins was, however, augmented by pre-treatment of the cells with actinomycin-D and by treatment of the harvests with deoxycholate or a combination of Tween-80 and ether.

Measles virus agglutinated fresh and formolized simian erythrocytes only and the titres were enhanced by pre-treatment of the erythrocytes with neuraminidase. Heterologous agglutinins for simian erythrocytes were found in all animal sera tested. Non-specific inhibitors removable by treatment with kaolin, acetone or a heparin-manganous chloride mixture occurred in cat, dog, ferret and rat sera.

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The pragmatic objective of biological research is effective control of living organisms for the fulfilment of human needs and the gradual elucidation of the basic processes of life. If "life" is defined in terms of cellular and subcellular organisation endowed with the propensity of genetic continuity through self-replication, then the study of viruses lies close to the heart of biology (Burnet , 1960).

Academic virology owes its unprecedented growth in the past two decades to the physical sciences. The development of the electron microscope resulted, inter alia, in the visualisation of the then enigmatic virus and its structural units and led inevitably to the recognition of similarities in basic physical parameters of viruses. Likewise, the development of methods of separation and characterisation of macromolecules provided the incentive for studies on antigenic similarities among morphologically-related viruses.

The study of cross-relationships between microorganisms is not intrinsically academic alone but has practical applications. The Weil-Felix test is the example "par excellence"; a serological aid for the recognition of antibodies to rickettsial infections of man, developed and standardised on the basis of an antigenic cross-reaction between two diverse genera of organisms, Rickettsia and Proteus. The

Jennerian concept of heterotypic vaccination, whereby cow pox virus is used to protect against smallpox, is founded likewise on antigenic similarities.

Serological interrelationships between viruses continue to be documented. Some have been studied in detail; for example, the pox viruses. None, however, presents so many unique and intriguing facets as the RNA triad of measles, rinderpest and canine distemper.

THE RNA TRIAD

Measles, rinderpest and distemper are enveloped RNA viruses with helical symmetry whose outer surface is fringed; features characteristic of myxoviruses. More precisely, the size and form of the nucleocapsid, the pleomorphic variation and the relative fragility of the virus particles indicate an affinity with the paramyxovirus sub-group of the myxovirus group typified by Newcastle disease virus. (Cruickshank, Waterson and Kanarek, 1962; Plowright, Cruickshank and Waterson, 1962; Waterson, 1962; Norrby, Friding, Rockborn and Gard, 1963a).

The three agents produce strikingly similar clinical signs and pathological changes in their natural hosts. The cytopathogenic changes they cause in cell cultures are also identical. These criteria reflect an intimate biological kinship and, therefore, the three viruses have been classified together tentatively as "medipest" viruses in the paramyxo group (Melnick and McCoombs, 1966).

HISTORY

MEASLES

Measles is an ancient and universal disease. The term "measles" seems to have originated from the Latin word 'misella', itself a diminutive of 'miser' meaning miserable. Langerhand in his 14th century poem "The vision of Piers the ploughman" used the term 'miser' to denote skin eruptions and sores (Wilson, 1962).

Rhazes, the celebrated Arab physician of the 10th century is credited with having clinically differentiated measles from smallpox but he and the Arab school regarded the two as diverse manifestations of the same malady. The concept of measles as an entity different from smallpox slowly emerged; in 1593 Kellwaye briefly alluded to the two as separate conditions in his treatise on plague and by 1629 the Parish Clerks of London listed the two diseases separately in the Annual Bills of Mortality.

The aetiology of measles remained obscure until 1911 when Goldberger and Anderson isolated a filterable agent from measles patients which, when inoculated into macaque monkeys, induced a measles-like syndrome. The role of a virus as the causal agent of measles was confirmed by Blake and Trask (1921a) and Rake and Shaffer (1940).

Measles virus grew in the chorio-allantois of the developing fowl embryo (Rake and Shaffer 1939; 1940) and in fowl embryo cell cultures (Plotz, 1938; Rake, Shaffer and Jones 1941). There was, however, no macroscopic or microscopic evidence of the replication of the virus and recourse to inoculation of susceptible monkeys was necessary. The golden era in measles virology began when Enders and Peebles (1954) developed a convenient in vitro system for the cultivation of the agent. They grew the virus in human tissues and obtained recognisable cytopathic changes which allowed quantitation of its biological and antigenic activities. Rapidly, thereafter, the ability of measles virus to haemagglutinate, haemadsorb and haemolyse was discovered (Mastyukova and Khait, 1960; Peries and Chany, 1960; Rosanoff, 1961). In vitro cultivation also yielded the large stocks of pure virus necessary for the elucidation of its ultrastructure (Waterson, Cruickshank, Laurence and Kanarek, 1961).

RINDERPEST

Rinderpest was and still is the most important pestilence of cattle. Maurer (1962) stated that it had had greater influence on the world's food supply than any other contagion of food-producing animals and the spectre still continued. It was the threat

to Europe's agricultural economy posed by rinderpest that led to the founding of the world's first veterinary school in Alfort in 1762. Similarly, international anxiety to contain rinderpest spurred the convening of the first of the world veterinary congresses in Hamburg in 1863 and the creation in France of the Office International Des Epizooties as a clearing-house for statistics of animal diseases (Vittoz, 1963).

The earliest accurate accounts of the disease were written in the 4th century A.D. (Barton, 1956). Its contagiousness and mode of transmission were defined at length by Ramazzini in 1711 and the filterability of the causal agent was demonstrated by Nicolle and Adil-Bey in 1902. Since then research on rinderpest has been concentrated on the development of live, modified strains of the virus for the prophylactic vaccination of livestock. Schein (1926), Edwards (1928) and Saunders and Ayyar (1936) successfully adapted virulent bovine strains of rinderpest virus to goats and noted that attenuation had occurred for cattle and water-buffaloes at different passage levels. Likewise, the serial passage of the bovine virus in rabbits (Cebe and Perrin, 1935; Nakamura, Wagatuma and Fukusho, 1938; Haddow and Idnani, 1947) and the bovine (Shope, Maurer, Jenkins, Griffiths and Baker, 1946; Daubney, 1951; Carter, 1952) and

lapinised viruses (Nakamura and Miyamoto, 1958) in fowl embryos led to the development of the further attenuated strains of the virus. Adaptation to golden hamsters, and white mice (Scott and Witcomb, 1958a) and sucking mice (Imagawa, 1965) have also been reported. The most exciting recent advance was the adaptation of the virus to cell cultures (Plowright and Ferris, 1957) which led to the realisation of similarities with measles virus (Plowright and Ferris, 1962a).

It merits emphasis that the biggest gap in our knowledge of rinderpest concerns the precise natural history of the virus. Elucidation of the natural history is perhaps the sine qua non for the final eradication of the disease.

CANINE DISTEMPER

Canine distemper, like measles, is a truly universal disease. Its origin is obscure. Laosson (1882) contended that the disease was known in the time of Aristotle. He also claimed that the epizootic that decimated canine species in Bohemia in 1028 was distemper and he listed 105 authors who, according to him, made a careful study of the disease. However, the consensus is that canine distemper akin to the clinical and pathological type seen to-day appeared only after the

discovery of the New World. Hensinger cited by Zuill (1895) thought that it came from Peru but Spinola cited by Whitney and Whitney (1953) and Henning (1956) opined that it originated in Asia. The latter view is the more acceptable because historical records reveal that there were no native dogs in South America before the arrival of the Spaniards (Whitney and Whitney, 1953).

Henning (1956) stated that the first authentic record of canine distemper was that of Ulloa who witnessed outbreaks of the disease during his extensive travels in South America between 1735 and 1746. The disease was imported into Spain in 1761 and from there it spread across Europe. Jenner (1809) recognized it for the first time in Britain and his accurate description of its several features is only matched by that of Blaine (1817).

Carre (1905) established the filterable nature of the causal agent of distemper, only seven years after the discovery of the first animal virus, foot-and-mouth disease virus, by Loeffler and Frosch (1898). Few other workers accepted Carre's findings until Laidlaw and Dunkin (1926) confirmed, beyond doubt, that the aetiological agent was a virus. Laidlaw and Dunkin (1926) also showed that the disease was naturally and experimentally communicable to ferrets; a finding that was to prove invaluable in later

studies on the pathogenesis of the disease. The ferret was the only experimental tool available until the advent of embryo cultivation procedures in the early nineteen-thirties. Even today, the inoculation of susceptible ferrets is still the most sensitive method for detecting canine distemper virus.

In 1948 McIntyre, Trevan and Montgomerie described a condition of dogs which they termed "hard pad disease" and which they differentiated from classical canine distemper. For well over a decade there was diagnostic confusion. One popular but much debated hypothesis was the existence of a plurality of distemper strains, one of which was the "hard pad" agent. (Verlinde, 1948; Innes, 1949; Larin, 1954; Larin and Hodgman, 1954). Subsequent studies revealed that classical distemper and hard pad disease were caused by antigenically and immunologically indistinguishable strains of distemper virus despite the disparities in their clinical and pathological features (Bateman, 1949, 1954; Welsh, 1949; Goret, 1950; Gillespie, 1951; Mansi, 1951, 1952; Cabasso, 1952; Haig, 1956). Admittedly, however, the suspicion of antigenic plurality among strains of distemper virus is not fully laid at rest.

Like research on rinderpest, research on canine distemper has been geared to the development of vaccines. The virus has been adapted to a variety of

heterologous hosts such as the ferret (Green, 1939; Green, Carlson and Surale, 1940), fowl embryos (de Monbreun, 1937; Mitschalich, 1938; Plummer, 1938; Haig, 1948, 1953; Cabasso and Cox, 1949, 1952), sucking mice (Morse, Chow and Brandly, 1953; Arakawa, Muto, Kaneko and Seki, 1959; Carlstrom, 1960) and sucking hamsters (Guttierrez and Gorham, 1955; Cabasso, Douglas, Stebbins and Cox, 1955). Then Rockborn (1959) demonstrated unequivocally specific sequential cytopathogenic effects of a mild strain of distemper virus in primary canine renal epithelial cells. Thereafter there was a surge of reports on adaptation of the virus to diverse cell cultures such as dog lung (Vantsis, 1959) ferret kidney (Hopper, 1959; Vantsis, 1959; Rao, 1963) bovine kidney (Bussell and Karzon, 1965_a) monkey kidney (Harrison, 1964) and stable human ammion cells (Bussell and Karzon, 1965_a).

EPIDEMIOLOGICAL LINKS

Differences between measles, rinderpest and canine distemper are best reflected in their natural hosts. Measles affects man and monkeys. Rinderpest is a natural infection of probably all, and only, artiodactyls especially buffaloes, cattle and pigs (Scott, 1959). Canine distemper occurs naturally in members of the families Canidae and Mustelidae (Gorham, 1960). It also attacks the raccoon

(Procyonidae) and the binturong (Viverridae). All three diseases are very contagious.

The clinical attack rate in measles is manifestly higher than any other infection in man. The source of infection is the infected patient. In virgin populations the susceptibility is absolute, a fact that clearly emerged from investigations of epidemics of the disease in the Cape York Peninsula in 1944 (Adels, Francis and Hajdusek, 1952) in southern Greenland in 1951 (Christensen, Schmidt, Bang, Andersen, Jordal and Jensen, 1953), in Canadian Indians and Eskimos in 1954 (Peart and Nagler, 1954) and recently in Kolyma, Russia (Elkin, 1961).

In high-risk populations the attack rate in susceptible members over one year old is usually 80 to 90 per cent. In urbanized communities those in the first 5-6 years of life bear the brunt of the attack and the age incidence curve registers two peaks, one for new entrants to school, i.e. when about 5 years old, and a lesser peak for their younger sibling contacts (Aycock and Eaton, 1925). According to the epidemiological data provided by Nicol (1956) about 40 per cent of susceptibles in a childhood population constitute the critical level for epidemic spread which thereafter progresses until 50 per cent of the susceptibles have been infected. In Britain the epidemiology of measles has a striking periodicity;

the highest incidence is usually between November and March and the interepidemic interval is generally two years (Butler, 1948). On the other hand, epidemics of measles occur every year in Nigeria and India and its devastating effects are attributed to malnutrition, low standards of hygiene and medical care.

The epizootic pattern of rinderpest in the 18th and 19th centuries was highlighted by the occurrence of the disease in the wake of major military campaigns (Gamjee, 1886; Dieckerhoff, 1890). Since then, trading in livestock has expanded and every epizootic of the disease in a previously disease-free country has been traced to the importation of domestic animals or game animals from enzootic foci (Sainte-Hillaire, 1865; Cilli, Mazzaracchoi and Roetti, 1951; Scott, 1957). For instance, Indian cattle shipped to Somalia sparked off the spectacular African panzootic in 1890 (Lugard, 1893; Hutcheon, 1902). Similarly goats from India during the Second World War were responsible for virgin epizootics in Ceylon and Malaya (Crawford, 1947; Orr, 1945). The more recent outbreaks in Zanzibar (Briant, 1956) and the Philippines (Villegas, 1960) were, likewise, precipitated by importation of cattle and buffaloes from territories where the disease was enzootic.

The rinderpest morbidity and mortality rate is generally very high in animals of all ages in virgin

epizootics. On the other hand, in enzootic regions, the disease picture is often confused because of varying degrees of innate resistance in the local stock and to the extensive use of live vaccines.

The epizootiological pattern of canine distemper in large cities has features similar to those of measles. Rockborn's studies (1958b, 1958c) revealed a seasonal prevalence in Stockholm, with the highest incidence in winter months. Similar data have been furnished by Hsiung and Stafseth (1952) and Ribelin (1953) for other temperate zones. No such information is available from the tropics.

The epizootiology of distemper is isolated, rural dog populations is also very reminiscent of that for measles. Virgin epizootics of distemper at Point Barrow and Anakbuvuk Pass in Alaska (Reinhard, 1953; Reinhard, Rauseh and Gray, 1955) and the Idaho outbreak in U.S.A. (Gorham, 1960) involved dogs of all ages and the mortality rates were high. In his excellent review of distemper, Gorham (1960) recorded a spectacular outbreak of the disease in a susceptible ferret colony. Most of the ferrets were kept in pens in groups of 5 or 6 and the rest were confined individually. The outbreak lasted for 64 days and the mortality was 100 per cent.

Like measles and rinderpest, distemper is a disease of the young, affecting most puppies before

they reach one year of age. Dogs, two years of age and older, are seldom attacked (Henning, 1956). Under natural conditions, the disease is transmitted by direct as well as indirect contact between diseased and healthy dogs.

Epizootiological studies on canine distemper in urban dog populations in Stockholm provide substantial proof of the theory of subclinical immunisation (Rockborn, 1958b, 1958c). Nearly 80 per cent of pups of unvaccinated bitches had neutralising antibodies until two months old. The percentage fell proportionately with increasing age; only 8 per cent of dogs, four to five months old, had antibodies. Rockborn, therefore, considered that this was the critical age group. Thereafter, the percentage of dogs having antibody titres progressively increased presumably because of active immunization from subclinical infection and Rockborn concluded that nearly 75 per cent of canine distemper infections in dogs in urban areas were subclinical.

The freedom of the infants from measles, the newborn calves from rinderpest and the young puppies from distemper is well documented. Infants under six months of age are generally considered to be refractory to measles and partially so in the next three months of life (Norrby, 1967). This refractoriness to the disease was attributed to the presence of maternally-

derived specific antibodies (Ruckle and Rogers, 1957; Bech, 1961) together with non-specific factors, the nature of which is not fully understood. (Anderson and Hamilton, 1949; Burnet, 1952). At birth, the infants have the same levels of measles antibodies as their mothers (Ruckle and Rogers, 1957; Bech, 1961; Strauss and Zeman, 1967). The antibodies waned in about six months (McCarthy, 1959; Strauss and Zeman, 1967). Twenty-six weeks after birth, 71 per cent of babies were sero-negative in the haemagglutination-inhibition test (Enders-Ruckle, 1964) and 93 per cent in the less sensitive complement-fixation test (Bech, 1961).

Unlike children who possess antibodies to measles at birth, calves born of dams immune to rinderpest did not have protective antibodies in their sera at birth. The antibodies were acquired by the ingestion of colostrum within two hours of sucking (Singh, Osman, El Cicy and Baz, 1967). The colostrally-derived passive immunity most marked in the first three months declined thereafter gradually and disappeared completely soon after weaning (Croveri, 1919; Henning, 1956). The persistence of the passive immunity was six to eight months (Montgomery, 1915; Tarantino, 1928; Jacotot and Colson, 1935). A few workers claimed that it lasted only two months but their conclusions were based on results obtained by the serum-virus simultaneous method of inoculation of calves (Askar, 1924; D'Costa

and Balwant Singh, 1933). The serum may have contained sufficient antibodies to suppress the thermal reaction expected after the inoculation of rinderpest virus and the absence of thermal reactions in the test calves was perhaps wrongly attributed to the possession of colostrally-derived immunity. The studies of Rabagliati (1924), Jacotot (1932), Hall (1933) and Carmichael (1934) in which virulent virus was used to test the immunity clearly showed that the immunity was substantial up to three months and progressively declined thereafter; a conclusion which was confirmed later (Brown, 1958a, 1958b; Singh et al., 1967).

The efficiency of maternally-derived immunity seemed to depend upon the titre of antibodies in the dam's serum at the time of calving which, in turn, depended upon the time and type of vaccination she received. Hale, Walker, Maurer, Baker and Jenkins (1946) observed that pregnant cows developed solid and lasting immunity to rinderpest avianized vaccine but there was no passive transfer of immunity to the progeny. On the other hand, Singh et al. (1967) demonstrated neutralizing antibodies to rinderpest in the colostrum and serum of two Friesian cows and two water-buffaloes vaccinated six to seven months prepartum with lapinized-avianized and caprinized virus strains respectively.

Unweaned calves from immune dams when inoculated with virulent (Hall, 1933; Daubney, 1934) or attenuated

virus (Gillam, 1944; Milne, 1956) either did not react at all or developed mild to moderate thermal reactions. In one study, death occurred in two calves aged seven and eight days inoculated with virulent virus (Carmichael, 1934). Reaction to active virus, however, was not evidence of development of immunity because a high percentage of calves from immune dams which reacted to inoculation received virulent or attenuated virus early in their lives, proved to be susceptible on re-infection several months later (Marque and Koumare Falley, 1949, 1950; Milne, 1956). The agreed hypothesis is that calves aged eight months or more from immune dames, can be successfully immunized with attenuated vaccines (Brown, 1958b). The recent observations of Singh et al. (1967) that approximately 80 per cent of bovine calves and 60 per cent buffalo calves born of immune dams had no circulating neutralizing antibodies demonstrable in tissue culture neutralization tests support the hypothesis.

Maternally-derived rinderpest antibodies had a half-life value of approximately 30 days (Brown, 1958b; Singh et al., 1967).

Carre (1903), after whom the malady is often named, was the first to observe that puppies from immune bitches were refractory to the disease. This was confirmed by Laidlaw and Dunkin (1926). Experimental proof for maternally-derived immunity to distemper was, however,

first provided by Slanetz (1935). Pups aged one week to one month were challenged with virulent distemper virus and no clinical disease ensued. Later, the pups were re-exposed to the virus; one group of pups which were inoculated first at one week and again at one month of age survived re-exposure while groups receiving a single inoculation at either one week or one month developed clinical distemper on re-exposure. Hoffman (1949) inoculated virulent distemper virus into nearly 4,000 dogs aged from one day to ten years. Pups from immune bitches survived the challenge until they were six weeks old. From the seventh week onwards the percentage susceptible increased markedly. A further comprehensive study of maternal immunity in distemper was reported by Gillespie (1956). In puppies from susceptible bitches, the attack rate in challenge infection was 100 per cent and 83 per cent of the pups, six to ten weeks of age, succumbed. In a group of pups, five to eight weeks of age and born of immune bitches, nine out of 23 developed clinical distemper and 9 per cent died. In a 12 to 16 week old group born of immune bitches the morbidity and mortality rates were 92 and 16 per cent respectively.

Gillespie (1956) and Ott, Gorham and Guttierrez (1957) demonstrated that the passage of maternal antibodies was mainly by way of the colostrum and to a lesser extent through the placenta. Gillespie, Baker, Burgher, Robson and Gilman (1958) later showed that

while the placental antibody usually protected pups up to two weeks of age the colostral antibody was the main source of the resistance up to six weeks of age. The mean serum titre of pups prior to sucking was about 3 per cent of the bitch's titre but after ingestion of colostrum the level of protective antibodies reached nearly 77 per cent of the bitch's titre.

The pup enters the urban dog population at a critical period when it has either lost, or is fast losing, its colostral immunity. The serological evidence presented by Hoffman (1949), Ott et al. (1955) and Rockborn (1958) in their studies on vaccinated and unvaccinated urban dog populations lends support to the presumption that on exposure to the disease most pups develop clinical or subclinical infection. The virus apparently persists from one generation of susceptibles to another, the long infective period contributing to a continual source of infection (Gorham, 1960).

SEROLOGICAL RELATIONSHIPS

Results of studies on the serological interrelationship between the measles, rinderpest and distemper viruses are not always in agreement largely because of differences in experimental designs and methods. For example, some laboratories used the natural and susceptible experimental hosts as sources of antibody whereas others used refractory hosts. This factor

alone accounts for some of the disparities in the results; antibodies from natural hosts are likely to show a broader spectrum of reactivity than antibodies from refractory hosts. Likewise, the preparation of antigens in diverse in vivo and in vitro systems was another variable in the experimental design. Others that had a considerable influence either on the experimental results or on their interpretation included differences in the time and temperature of incubation and in the delineation of the end-points. Nevertheless, the bulk of the evidence points towards the occurrence of one-way crosses between the three viruses (Warren, 1960; Bussell and Karzon, 1966; Imagawa, 1968).

Measles and distemper: Four decades ago, Bryan (1928) implicated canine distemper virus as the cause of the then widely prevalent but undiagnosed acute respiratory illness of man. He claimed to have developed a severe form of "la grippe" after attending a dog show and to have successfully transmitted the agent from his blood to four puppies. The puppies allegedly developed a clinical syndrome not unlike that of distemper. He presented details of four additional outbreaks involving 11 children all of whom had previous contact with "distemper-infected" dogs.

Likewise, Nicolle (1931), Vuori (1946) and Kirk (1947) wondered whether there was an epidemiological link between the human disease and canine distemper. Nicolle

(1931) reported inapparent infections in man following contact or inoculation with distemper virus and he postulated that man was the reservoir host for distemper. His reasoning was that if a disease was overt in one species and inapparent in another, then it was older in and probably derived from the latter species.

Adams (1941) described a new form of epidemic pulmonary disease in prematurely-born and young infants which was highly contagious and was characterized by features such as moderate to severe rhinitis, pharyngitis, cough, dyspnoea and biphasic fever; features that are common to distemper. The pathology of the human disease was also strikingly similar to that of distemper, the chief lesions being proliferation and destruction of alveolar lining cells, preponderance of mononuclear cells in the inflammatory exudates and the presence of cytoplasmic inclusions in bronchial epithelial cells. Adams' report and the subsequent detailed patho-histological studies of Pinkerton et al. (1945) on Hechts' giant-cell pneumonitis of infants and distemper of mink, ferrets and foxes prompted a survey of human sera. Soon a spate of reports of adults possessing antibodies reactive with both measles and distemper viruses appeared in the literature (Adams, 1953; Imagawa et al., 1954, 1960; Carlstrom, 1955, 1956, 1957; Karzon, 1955; Prier, et al., 1956; Hopper, 1959; Bech, 1960; Goret, et al., 1960). The antibody

link was recognized much earlier than the morphological relationship (Cruickshank et al., 1962; Norrby et al., 1963a).

Most human sera acquired the capacity to neutralize canine distemper virus early in adolescence. This was first convincingly demonstrated by Imagawa et al. (1954) who conducted neutralization tests in ovo, using the egg-pathogenic Lederle strain of distemper virus. Samples of human sera from different age groups including prematurely-born infants and also samples of human gamma-globulin had as high titres of neutralizing antibodies as in the reference hyperimmune ferret serum; the latter gave 100 per cent protection to ferrets in a dilution of 1:640 against a challenge dose of 100 MLD of ferret-passaged virus.

Karzon (1955) examined the neutralizing ability of 266 human sera against 20 to 50 EID_{50} of distemper virus. Human gamma-globulin was equal to hyperimmune canine distemper serum in protective value. Antibodies to distemper virus in man ranged from 1:5 to 1:640 with a geometric mean of 1:40. Karzon also observed that the antibody was transmitted in utero, was lost in children about six months old and reappeared at two to ten years of age. The incidence in the adult population was nearly 100 per cent.

Karzon (1955) did not find any correlation between the antibody titres and a past history of any specific

infection, including measles. Such a relationship was, however, clear cut in the serological investigations of Carlstrom (1956, 1957) in Sweden. Her neutralization test was performed in the brains of sucking mice using a mouce-adapted strain of distemper virus. In patients who had a history of measles the incidence of distemper neutralizing antibodies was about 90 per cent (Carlstrom, 1956). Further, in the course of an epidemic of measles in Stockholm in 1957, she demonstrated a four-fold rise in antibodies neutralizing distemper virus between acute and convalescent phase serum in 11 of 14 patients. The titres ranged from 1:5 to 1:625 (Carlstrom, 1957). In another study, the ratio of the titres of distemper antibodies to those of measles antibodies was 1:25 or greater (Carlstrom, 1959a, 1959b).

The serological surveys of Bech (1960) in Greenland and Black and Rosen (1962) in Tahiti have abundantly confirmed the aetiological relationship of measles virus with the presence of antibodies in human sera reactive with distemper antigens. Bech (1960) who investigated an extensive outbreak of measles in an isolated community in Sukkertoppen in 1955, used the measles and distemper complement-fixing antigens to detect the two types of antibodies. There was no canine population in the community. Antibodies to distemper were detected in four of five patients in titres 8 to 32 times lower than the titres to measles antigen. Four years later, the

measles antibodies had dropped to levels identical with, or only two-fold higher, than those of distemper antibodies which remained stable. Antibodies reactive to distemper antigen were not detected in all measles patients although some of them had high titres to measles (Bech, 1960).

Canine distemper was unknown in Tahiti before 1956 when an explosive epizootic appeared killing nearly two-thirds of the canine population. Measles epidemics had occurred in 1922, 1950-51 and 1960. Black and Rosen (1962) investigated the 1960 outbreak. Measles and distemper antibodies were detected in persons above 11 years of age but not in children that were born after the 1951 epidemic. A cell culture neutralization test was employed to identify the antibodies. Black and Rosen also noted that individuals nine years of age and older who were born before the distemper outbreak carried both measles and distemper antibodies although they were exposed only to measles. Those who were not exposed to measles had neither measles nor distemper antibodies although they were exposed to distemper. Sera samples collected eight years later revealed no change in measles antibody titres but a two-fold increase in the distemper antibody titres. They concluded that the difference in values was not due to re-exposure of the population to distemper virus but to slower decay of the distemper antibodies.

Millian, Maisel, Kempe, Plotkin, Pagans and Warren (1960) screened the sera of children who had no prior history of measles for canine distemper neutralising antibodies. Thirty-six children aged between 2 to 12 years were then divided into three groups of 5, 26 and 5 and given formaldehyde-inactivated distemper vaccine, live egg-adapted distemper vaccine, and live attenuated measles vaccine respectively. The prevaccination distemper antibody titres in the children ranged from 1:10 to 1:60 and the measles complement-fixing antibody titres were less than 1:16. Three to four weeks following distemper vaccination the distemper antibody titres rose to 1:332 with a geometric mean value of 1:100. There was no increase of measles complement-fixing antibody titres.

In three of five children inoculated with live measles vaccine the distemper antibody levels were comparable to those elicited by live canine distemper vaccine in other children. Measles antibody titres increased to 1:32 in only two; the corresponding distemper antibody titres were high.

As in man, the response of the monkey to measles virus is characterized by the appearance of antibodies to both viruses, the heterologous response being somewhat delayed and of a lower order (Schwarz, Boyer, Zirbel and York, 1960; Karzon, 1962). On the other hand, the administration of live egg-adapted distemper virus failed to produce measles antibodies (Schwarz et al., 1960; DeLay, Stone, Karzon and Enders, 1965).

The homologous antibody response was high in the studies of Schwarz et al. (1960) but DeLay et al. (1965) obtained no response. They empoyed the virulent Snyder Hill strain of virus to inoculate three monkeys whereas Schwarz et al. (1960) administered the attenuated egg-adapted strain into their six experimental monkeys.

Sera from rabbits, guinea pigs, ferrets and fowls exposed to measles virus often contained moderate to good titres of measles antibodies but only low titres of distemper antibodies in a low percentage of the animals (Imagawa, Goret and Adams, 1960; Bussell and Karzon, 1966). Even hyperimmunization with live measles virus elicited only a low level distemper antibody response in rabbits and guinea pigs (Bussell and Karzon, 1966) and in fowls (Cabasso, Kiser and Stebbins, 1959). In the mouse neutralization test, sera from ferrets inoculated with measles virus effectively neutralized the mouse-adapted strain of distemper virus. The serum, however, conferred no passive protection in ferrets against challenge with ferret or canine virus although the incubation periods of the disease were longer (Adams and Imagawa, 1957a, 1957b).

Neutralization of measles virus by distemper antibody has been difficult to demonstrate. Bardach, Cross-Decam and Goret (1947) and Goret (1961) used hyperimmune canine distemper serum and failed to neutralize their strains of measles virus allegedly adapted to dogs. Cabasso et al. (1959) immunized

puppies and chicks with avianized distemper virus and demonstrated high levels of homologous antibodies in both species. The sera from the puppies contained neither complement-fixing nor neutralizing antibodies reactive with measles virus whereas the chick sera had complement-fixing antibodies but not neutralizing antibodies to measles virus.

Karzon (1962) hyperimmunized chickens and rabbits with distemper virus and obtained homologous neutralization titres ranging from 1:128 to 1:1024 but the sera failed to neutralize measles virus in cell culture. On the other hand, employing a mouse-adapted strain of measles virus, Carlstrom (1958) demonstrated the neutralizing ability of rabbit anti-distemper serum. The neutralization test was performed in the brains of sucking mice. Arakawa et al. (1959) and Carlstrom (1962) confirmed and amplified these findings. The latter also demonstrated that in the mouse-neutralization test the distemper serum gave much higher titres of antibody reactive with measles virus than in the cell culture neutralization tests (Carlstrom, 1962).

According to Imagawa et al. (1960) distemper antisera prepared in guinea pigs failed to neutralize measles virus in cell culture whereas hyperimmune ferret serum neutralized in low titres. The latter finding is somewhat surprising because it has been reported that dogs hyperimmunized with different strains of distemper

virus possessed little or no neutralizing antibodies to measles virus (Karzon, 1962; DeLay et al., 1965; Roberts, 1965). Likewise, cattle and monkeys inoculated with distemper virus developed no antibodies to measles virus (Schwarz et al., 1960; Karzon, 1962; DeLay et al., 1965).

The response of the dog to measles virus has been the subject of a series of investigations. The earliest study was probably that initiated by Bardach et al. (1947) in 1938. Their claim that measles virus was serially passaged in puppies in which it produced reproducible clinical and pathological features was not founded on virological or serological evidence. Equally unconvincing are the reports of Rjazantseva (1956) and Goret (1961) of transmission of measles virus into puppies; they neither attempted recovery of the virus from their experimental puppies nor did they establish the presence of measles antibodies by reliable serological tests.

In the early sixties Gillespie and Karzon (1960) and Warren et al. (1960) independently demonstrated that a single inoculation of live measles virus elicited the production of fair levels of measles antibodies and low but recognizable levels of distemper antibodies. The presence of measles antibodies, irrespective of the titre, reflected a state of immunity to distemper as evidenced by the results of challenge infection

(Gillespie and Karzon, 1960). Confirmatory as well as contradictory reports appeared later. Moura and Warren (1961) demonstrated that two different strains of measles virus injected into dogs by different parenteral routes did not cause clinical signs or the development of distemper antibodies. Measles antibodies, however, appeared and were recognized by complement-fixation and neutralization tests.

Roberts (1965) did not detect measles neutralizing antibodies in dogs hyperimmunized with distemper virus. On the contrary, antibodies to distemper virus readily developed in dogs after the second inoculation with measles virus. When two groups of dogs hyperimmunized with measles and distemper viruses were reinoculated with the heterologous viruses there occurred an anamnestic response which was both homotypic and heterotypic. To demonstrate the presence of cross-reacting antibodies, Roberts adsorbed the sera of measles-hyperimmune dogs challenged with distemper virus with each purified virus preparation. The antibody that was eluted from the virus-antibody complex was then tested for its ability to neutralize the heterologous virus. Using this technique he demonstrated that the antibody induced by measles virus did neutralize distemper virus but the reverse was not true. He therefore postulated that the two viruses were related through their nucleocapsid antigen(s) which were probably similar and that

there were no common envelope antigens. This hypothesis which is not based on experimental evidence might sound convincing if considered together with the earlier findings of Cabasso, Avampato, Kiser and Stebbins (1960) that measles and distemper immune sera did not inhibit the growth of the heterologous virus in cell culture. Furthermore, Warren et al. (1960) used six different strains of distemper virus and demonstrated equivocal or low measles complement-fixing antibodies in some of their experimental pups. Challenge with virulent distemper virus several days later considerably enhanced the antibody titres. A finding which lends support to Roberts' hypothesis of the sharing of nucleo-capsid antigens between the two viruses.

Further evidence of the close antigenic relationship between measles and distemper, particularly the distinct one-way cross, stems from the immunocytological studies of Connolly, Allen, Hurwitz and Millar (1967) and Connolly (1968). Using measles and canine distemper conjugated sera they observed measles antigen in sections of brain from fatal cases of subacute sclerosing panencephalitis with the homologous but not the heterologous serum (Connolly et al., 1967). On the other hand, the development of distemper virus antigens in fowl embryo fibroblast cultures and the presence of the antigens in frozen sections of brain from a case of distemper encephalitis were readily demonstrable both with the homologous and

heterologous conjugated sera (Connolly, 1968); further evidence of the one-way cross.

Measles and rinderpest: Resemblances in the natural pathology of measles pneumonitis in infants and distemper in pups were already known (Pinkerton et al., 1945; Adams, 1953) when Thiery (1956) first published his account of the histopathology of natural rinderpest. Thiery demonstrated syncytia and cytoplasmic inclusions in the lymphoid tissues and epithelial cells of the mucosa of the upper part of the alimentary tract of rinderpest-infected cattle. Three years later, it was announced that adult human sera contained antibodies capable of neutralizing rinderpest virus (Plowright and Ferris, 1959a). This marked the end of the era of speculation about the postulated kinship between the viruses of measles, rinderpest and distemper (Koprowski, 1958; Imagawa et al., 1960; Warren, 1960).

Human antibody patterns to rinderpest did not correlate with a past history of measles infection as well as the distemper antibody patterns (Bech, 1959; Black and Rosen, 1962). Sera from 16 of 18 adults on the staff of a rinderpest research laboratory neutralized rinderpest virus to titres between 0.6 and 1.9 log dilution. The two negative cases had no history of measles infection (Plowright and Ferris, 1959a). In the course of an epidemic of measles among children in a rinderpest-free area the acute and convalescent phase

sera of four cases had rinderpest antibody; the first sample was obtained five days after the onset of clinical illness. Nine of 17 children developed measles and rinderpest antibodies during convalescence, the homologous antibody levels being the higher (Plowright, 1962a). The heterologous antibody titre was enhanced by the addition of guinea pig complement (Plowright, 1962a), a finding reminiscent of that reported with neutralization of measles virus by distemper serum (Adams and Imagawa, 1957).

No children immunized with live attenuated measles vaccine, both measles and rinderpest neutralizing antibodies were detected to a low level on or about the twelfth day of 1 of 11 cases. On the thirtieth day, homologous antibody had developed in all, the titres ranging from 1:80 to 1:640 in 10 and 1:20 in the eleventh case. In contrast, only 7 to 11 children had rinderpest antibody; the titres ranged from 1:10 to 1:20 (Imagawa et al., 1960).

Monkeys inoculated with measles virus responded with high levels of the homologous antibody and low levels of rinderpest neutralizing (Plowright, 1962a; DeLay et al., 1965) and complement-fixing antibodies (DeLay et al., 1965).

There are only two published reports in the available literature on the behaviour of measles virus in cattle and the data disagree. DeLay et al. (1965)

inoculated six cattle subcutaneously with 8.0 ml of cell cultured measles virus and obtained no antibodies reactive with measles, rinderpest or distemper viruses at 24 to 30 days after inoculation. Unfortunately, they did not examine later serum samples for antibodies. Provost, Borredon and Maurice (1967) examined the sera of six calves given measles virus for measles and rinderpest antibodies using the haemagglutination-inhibition test and the rinderpest virus neutralization test in cell culture. There was no response in one calf. The others developed neutralizing antibodies at three weeks, the titres ranging from 1:32 to 1:128. Only two calves had haemagglutination-inhibition antibodies in titres of 1:2 and 1:16 respectively. At nine weeks, four calves had moderate to high neutralizing antibody titres ranging from 1:64 to 1:1024 but equivocal, poor or moderate haemagglutination-inhibition antibody titres ranging from 1:2 to 1:32. The fifth calf died of other causes. A concomitant measles neutralization test was not carried out and therefore the measles-specificity of the haemagglutination-inhibiting antibodies was not established.

Dogs inoculated with measles virus developed low levels of rinderpest neutralizing antibodies in 6 of 13 cases. The neutralization test was performed in rabbits using 10 ID_{50} lapinized-rinderpest virus (DeLay et al., 1965).

Cattle infected with virulent or vaccine strains of rinderpest virus contained antibodies reactive with measles virus. They were demonstrable by the neutralization test (Imagawa et al., 1960; Plowright, 1962a) and by the haemagglutination-inhibition test (Waterson et al., 1963; Bogel, Provost and Enders-Ruckle, 1966a, 1966b; Rowe, Zwart and Kouwenhoven, 1967). Calves immunized with rabbit or egg-adapted rinderpest virus developed measles and rinderpest neutralising antibodies, the homologous titres being the higher (Imagawa, 1968). In one study, the heterologous or measles titres were higher than the homologous rinderpest titres (Plowright, 1962a); a result ascribed to the greater sensitivity of the measles neutralization test (Bussell and Karzon, 1966).

The relationship between the two viruses has been further delineated by data from immuno-diffusion (Provost and Borredon, 1968) and immuno-cytological tests (Leiss, 1963). In their studies on an intracellular haemagglutinin in rinderpest cell cultures Provost and Borredon (1968) detected two antigens, one of which induced haemagglutination of monkey erythrocytes and another which was adsorbed on to the erythrocytes without producing agglutination. The latter antigen was thermolabile and was inactivated by ether. It produced a band of precipitation with measles serum. The precipitation reaction, however, was not identical with that induced by measles haemagglutinin with the

same serum (Provost and Borredon, 1968). Measles-infected cells showed specific fluorescence when treated with rinderpest-immune globulin from hyperimmune cattle sera (Leiss, 1963). Both studies await confirmation.

Distemper and Rinderpest: A link between rinderpest and distemper was first hinted by Polding, Simpson and Scott (1959) on the basis of an earlier astute observation that dogs reared in an area enzootic for rinderpest enjoyed apparent freedom from distemper probably from feeding over long periods on meat from rinderpest-infected goats (Polding and Simpson, 1957). The lead was vigorously pursued by several workers and the literature on the subject was reviewed in detail by Mornet, Goret, Gilbert and Goueffon (1960) up to that time.

Dogs inoculated with virulent rinderpest virus developed either a subclinical disease (Hallen, McLeod, Charles, Kerr and Jan, 1871; Curasson, 1932; DeLay et al., 1965) or a mild febrile response (Morcos, 1931; Polding et al., 1959). Virus was recovered from the blood (Morcos, 1931; Polding et al., 1959; Mornet et al., 1960) and the faeces (Viseur, 1771) by sub-inoculation into susceptible calves. Unequivocal proof of proliferation of the virus in dogs is lacking. Rinderpest virus elicited a homologous antibody response but did not stimulate the production of distemper antibodies (Polding and Simpson, 1957; Scott and Brown, 1958; Mornet et al., 1960;

DeLay et al., 1965) nor measles antibodies (DeLay et al., 1965). The antibodies were demonstrated either by complement-fixation (Polding and Simpson, 1957; DeLay et al., 1965) or neutralization tests (Scott and Brown, 1958; Mornet et al., 1960; DeLay et al., 1965).

Dogs inoculated with virulent distemper virus developed good levels of rinderpest complement-fixing antibodies and poor levels of neutralizing antibodies. Prior sensitization of the dogs with cell cultured measles virus did not influence the titres (DeLay et al., 1965).

Convalescent and hyperimmune rinderpest serum derived from cattle neutralized avianized (Goret et al., 1959; Mornet et al., 1960) and ferret distemper virus in vitro (Goret et al., 1959) in dilutions similar to those of homologous sera with mouse-adapted distemper virus the neutralization titre of the homologous serum was higher, (Imagawa et al., 1960). Rinderpest hyperimmune serum derived from rabbits had no neutralizing activity on avianized distemper virus (Mornet et al., 1960). Likewise, DeLay et al. (1965) failed to show neutralization of cell-cultured distemper virus with hyperimmune rinderpest serum from cattle and rabbits.

Passive protection studies in dogs (Polding et al., 1959) and ferrets (Goret et al., 1960c) revealed that rinderpest hyperimmune cattle serum conferred no protection when administered simultaneously with virulent

virus.

Imagawa (1968) considered the reported results of neutralization tests paradoxical. It is, however, clear that it was the sensitivity of the indicator host that had influenced the results; the tests conducted in mice and the fowl embryoes gave reproducible results.

Administration of distemper virus to monkeys and cattle provoked an homologous antibody response; rinderpest antibodies were detected only in the former species in low titres (DeLay et al., 1965).

The antigenic relationship between distemper and rinderpest viruses has also been evident in agar gel diffusion studies (White et al., 1961; Fraser, 1966). A line of identity appeared when both antigens were diffused against hyperimmune canine distemper serum but spur formation occurred when rabbit hyperimmune rinderpest serum was used (White et al., 1961). The latter finding was not confirmed by Fraser (1966). Using rabbit hyperimmune rinderpest serum he obtained a distinct line of identity with hyperimmune canine distemper serum against infected mink and dog tissue antigens. In comparisons of the two viruses White et al. (1961) found no viral antigen in non-infected animal organs. A similar comparison was not reported by Fraser (1966).

IMMUNOLOGICAL RELATIONSHIPS

Measles and distemper: Inoculation of measles virus into

dogs usually induced a resistance to distemper irrespective of the distemper antibody response (Warren et al., 1960; Moura and Warren, 1961; Slater and Murdock, 1963; DeLay et al., 1965). Moreover the resistance developed within three days after inoculation of pups with a high dose of measles virus (Anon, 1964). Inoculated pups have withstood challenge with virulent distemper virus administered intracerebrally, intravenously, intranasally and subcutaneously (Gillespie and Karzon, 1960; Moura and Warren, 1961; Slater and Murdock, 1963; DeLay et al., 1965) but a high titre of measles virus was generally considered necessary to stimulate resistance in dogs to intracerebral challenge infection with the virulent Snyder Hill strain of distemper virus (Gillespie and Karzon, 1960; Slater, 1966). Furthermore, measles virus was capable of inducing resistance in pups having significant levels of maternally-derived distemper antibodies (Moura and Warren, 1961). A significant degree of resistance was also stimulated by inactivated measles vaccine (Warren et al., 1960). The resistance induced by live measles virus lasted up to six months (Gillespie and Karzon, 1960).

The mechanism underlying the heterotypic resistance in dogs receiving measles virus has been the subject of considerable speculation and controversy. Slater and Murdock (1963) postulated that a state of "selective

cellular blockage" was induced by measles virus by modifying the susceptibility of cells in target organs. They presumed that a limited population of unmodified cells must be available for distemper virus to establish itself and produce clinical disease. The mechanism of the so-called blockage, however, was not explained.

The role of viral interference and interferon production in measles-induced resistance to distemper was discussed by Peacock (1966). He considered the formation of incomplete virus and the production of endogenous interferon as likely events in the development of the heterotypic resistance. He cited the findings of Wagner (1960) that influenza virus in persistently infected cell cultures stimulated the production of new virus progeny or interferon or both and that the persistent low-grade infection was probably the result of an equilibrium between the two antagonistic products of cell infection. Peacock (1966) therefore wondered if such an equilibrium existed in the tissues of dogs inoculated with live measles virus. Moreover, because measles virus has not been reisolated from inoculated dogs (Moura and Warren, 1961; Slater and Murdock, 1963) the equilibrium between measles virus and interferon production in the canine tissues might be such that new virus progeny were likely to have the characteristics of incomplete virus (Peacock, 1966).

A common explanation for the measles-induced

resistance to distemper was that dogs were sensitized and that exposure to distemper virus stimulated a rapid anamnestic antibody response to both viruses (Warren et al., 1960; Anon, 1964; Roberts, 1965; DeLay et al., 1965). The explanation supported the assumption that the induced resistance was not due to a pre-existing humoral antibody but was related to cellular immunity (Moura and Warren, 1961).

Slater (1966) disagreed with the hypothesis that immunity to distemper infection in dogs pre-sensitized with measles virus was mediated by a quick anamnestic response. In one experiment where two groups of dogs were repeatedly sensitized with inactivated measles vaccine or live distemper vaccine intracerebral challenge with virulent distemper virus was followed by death in the first group and survival in the other. Slater (1966) contended that if the resistance was attributed to an anamestic response it should have occurred in both groups. He did not give the antibody titres in the two groups of dogs. In another study, several litters of dogs were inoculated intravenously with $10^{8.0}$ TCD_{50} of measles virus and challenged intracerebrally 12 hours later with Snyder Hill distemper virus. The test dogs remained well whereas all the controls were dead within seven days. Examination of serum samples collected from the controls just prior to death and at the same time from the test dogs showed no distemper antibodies at a

dilution of 1:2. Slater (1966) considered the data invalidated the hypothesis that resistance to distemper infections in dogs previously inoculated with measles virus was mediated by an anamnestic antibody response to distemper.

The behaviour of ferrets to measles virus is enigmatic. A large series of experiments in ferrets were conducted by Imagawa and his associates between 1953 and 1958 in studying the immunological relationship between measles and distemper. They concluded that ferrets were difficult to immunize with measles virus; most ferrets inoculated with measles virus succumbed to challenge infection with distemper virus; a few showed prolongation of the incubation period, modification of the clinical illness and the duration of disease. In one study 9 of 21 ferrets survived the challenge (Imagawa, Yoshimori and Adams, 1953; Imagawa, Wright and Adams, 1954; Imagawa and Adams, 1957; Adams and Imagawa, 1957a, 1957b, 1958; Adams, Imagawa, Chadwick, Gates and Siem, 1959). Apparently, the mechanism of anamnestic antibody response failed in this system. Cabasso et al. (1960) demonstrated measles antibodies in ferrets given repeated doses of measles virus-adjuvant mixtures. There was no distemper antibody response. On challenge with virulent distemper, the ferrets proved to be fully susceptible.

Protective trials against measles in human subjects using distemper virus as the immunizing agent are few in number. In one carried out in a mental institute

Adams, Imagawa, Wright and Tarzan (1959) inoculated 200 persons of unstated age with 1.0 ml of the live Lederle strain of egg-adapted distemper vaccine. The incidence of measles in the next five years was 1.8 per cent in 165 vaccinated subjects and 5.9 per cent in 1190 unvaccinated controls.

In the course of a measles outbreak in Panama distemper avianized vaccine was given to 388 persons of different age groups. The clinical attack rate of measles was 3.6 per cent in the vaccinated group and 6.0 per cent in the controls (Hoekenga, Schwarz, Palma, Carrizo and Boyer, 1960). No antibody determinations were reported and the difference in the clinical attack rate in the vaccinated and unvaccinated groups was not significant.

Measles and rinderpest: There is no modern published report of the behaviour of rinderpest virus in man. Monkeys inoculated with the virulent Kabete "O" strain of rinderpest virus usually developed complement-fixing and neutralizing antibodies to rinderpest. Complement-fixing antibodies to measles virus were demonstrated in one animal only. The results of challenge infection with measles virus were vitiated by the controls failing to develop marked clinical signs. Challenge inoculation stimulated measles complement-fixing antibodies in five of nine monkeys (DeLay et al., 1965).

In the studies of Plowright (1962a) and DeLay et al.

(1965) cattle were serologically refractory to measles virus and the animals succumbed to challenge infection with virulent rinderpest virus. Provost et al. (1967), on the other hand, obtained haemagglutination-inhibition antibodies reactive with measles virus and neutralizing antibodies to rinderpest virus following a single inoculation with the MB-113Y strain of measles virus adapted to grow in bovine cell culture (Schwartz and Zirbel, 1959). The inoculated cattle developed a febrile response characterized by hyperthermia on the fifth and sixth day after inoculation when virus was recovered from the leukocytic fraction of the blood. The cattle resisted challenge infection with virulent rinderpest virus.

Rabbits given three or more inoculations of measles virus by various routes, with and without adjuvants, developed neutralizing antibodies to rinderpest. They withstood challenge with lapinized rinderpest virus (Plowright, 1962).

Distemper and rinderpest: Cross-protection studies in susceptible natural and experimental hosts of rinderpest and distemper viruses revealed a one-way cross similar to the measles-distemper relationship, the rinderpest virus being the "prime" antigen (Warren, 1960).

Polding et al. (1959) successfully immunized two dogs with a high dose of the Kabete "O" strain of virulent rinderpest virus. There was viraemia. High

titres of neutralizing antibodies to rinderpest were detected three weeks after inoculation. Both animals resisted a distemper challenge. On the other hand, hyperimmune rinderpest serum given in a dose of 20 ml failed to protect any of five dogs exposed to distemper (Polding et al., 1959).

The behaviour of ferrets to rinderpest virus was erratic. In one study of five of six ferrets inoculated with the virulent Senegal strain of rinderpest virus were found to be immune on challenge with distemper virus (Goret et al., 1958). A finding confirmed by Mornet et al. (1959a) in one study but not in a subsequent experiment (Mornet et al., 1960). Both groups of workers were successful in immunizing a large number of ferrets with lapinized rinderpest virus (Goret et al., 1958; Mornet et al., 1959a). Later it was observed that the immunity conferred by lapinized rinderpest virus lasted for nearly a year (Goret et al., 1960b).

Attempts at immunization of cattle against rinderpest with distemper virus were not always successful. Goret et al. (1958) and Mornet et al. (1960) reported success with virulent ferret-derived and attenuated avianized strains of distemper virus. Protection seemed to be dose-dependent (Mornet et al., 1959a, 1959b). There was also a direct relationship between the development of distemper antibodies in cattle inoculated with distemper virus and their resistance to challenge

infection with rinderpest virus (Gilbert et al., 1960). In contrast, Polding et al. (1959) and DeLay et al. (1965) failed to obtain resistance to rinderpest in their experimental cattle by inoculation with distemper virus. Polding and his colleagues even tried multiple inoculations of the virus without success while in DeLay's experiments although five of six cattle had distemper antibodies at the time of challenge, they all succumbed to rinderpest.

Rabbits were not protected against rinderpest even with multiple doses of virulent or avianized distemper virus (Goret et al., 1960a; Mornet et al., 1960; Plowright, 1962a). However, distemper sera prepared in the dog, ferret, horse and cattle neutralized lapinized rinderpest virus, albeit to low titres (Polding and Simpson, 1957; Goret et al., 1960a; Mornet et al., 1960; Villemot et al., 1961).

SUMMARY

Infection and immunization of man and monkeys with measles virus engendered antibodies reactive with measles, rinderpest and distemper viruses. Measles virus immunized against human and simian measles, bovine rinderpest and canine distemper but the protective value of rinderpest and distemper viruses against measles remains to be established.

Rinderpest-convalescent cattle had antibodies which

neutralized measles, rinderpest and distemper viruses. Rinderpest virus immunized cattle against rinderpest and the dog against distemper. Its effect upon man and monkey is unknown. Both measles and distemper viruses immunized cattle against rinderpest.

Canine distemper convalescent serum reacted with canine distemper and rinderpest virus but equivocally with measles virus. Canine distemper virus immunized dogs against distemper, cattle against rinderpest and, probably, man against measles. Canine distemper was prevented by measles and rinderpest viruses.

The three viruses shared similar or closely related antigens but their natural hosts were dissimilar. Experimentally, however, the three agents have been propagated in many identical host systems, the most important of which is the sucking mouse. Adaptation of the "medipest" viruses to the brain of the sucking mouse entailed the loss of their strict zootropic propensity. The disease induced by the three viruses in infant mice was clinically and pathologically indistinguishable (Imagawa, 1965, 1968). The mouse-adapted viruses were differentiated only on the basis of their relative avidity in serological reactions. Each virus was generally neutralized in higher titre by its homologous antibody (Imagawa, 1968). When re-introduced into their natural hosts, the zootropism was regained, measles being able to infect only the monkey, rinderpest only cattle

and distemper only the dog.

HOST RANGE

MEASLES

Natural infections: Measles as a natural disease is restricted to the primates. The simian disease, naturally occurring or artificially induced, closely parallels human measles in clinical and pathological features but is generally mild or subclinical (Blake and Trask, 1921a, 1921b, 1921c; Shaffer, Rakes, Stokes and O'Neil, 1941). Wild free-living monkeys appear to be free of the disease. Infection is gained through human agency (Meyer, Brooks, Douglas and Rogers, 1962; Bech, 1962; Zhdanov, 1962). The agent known as "MINIA", originally isolated from captive monkeys, is measles virus (Rhodes and van Rooyen, 1968).

Experimental infections: Measles virus has been successfully propagated in fowl embryos (Rake and Shaffer, 1939, 1940; Zhdanov and Fadeyava, 1956). Milovanovic, Enders and Mitus (1957) used the Edmonston strain of the virus, previously adapted to grow in human amnion cells, to infect six-day old embryos by the amniotic route and evidence of viral replication in amniotic and allantoic fluids was furnished by back titration in human amnion cells. Neither haemagglutinins nor complement-fixing antigens were detected in the tissues or fluids of the fowl embryos. The embryos themselves were unharmed.

Mouse adapted strains of measles virus are now

available (Imagawa and Adams, 1958; Carlstrom, 1958; Matumoto, Saburi, Aoyama and Mutsi, 1964). The pathology of measles in the brain of the sucking mouse is indistinguishable from that produced by mouse-adapted strains of rinderpest and canine distemper viruses (Imagawa, 1965). The mouse-propagated measles virus, however, retained its ability to evoke clinical disease in monkeys and its characteristic cytopathology in cell culture.

Most claims of transmission of measles virus to rabbits (Hardi, 1921; Nevin and Bittman, 1924, 1925; Grund, 1922; Scott and Simon, 1922, 1924) and to guinea-pigs (Dual and d'Aunoy, 1922; Taniguchi, Hosokawa, Kuga and Terada, 1935) are based on dubious or unconfirmed experimental data. In reviewing the literature on the subject Wilson and Miles (1955) mentioned that such reports were severely criticised by Purdy, 1925. McCartney (1930) re-investigated the issue and came to the conclusion that the lower mammals are refractory to the virus.

Measles virus apparently does not induce a clinical or sub-clinical state in dogs and ferrets. Warren, Nadel, Slater and Millian (1960), Gillespie and Karzon (1960), and Moura and Warren (1961) all expressed the view that restricted multiplication of the virus occured in canine tissues. Their evidence was serological and not virological.

Goret (1961), likewise, did not isolate the virus from dogs. He inoculated dogs with blood from measles patients and witnessed a clinical syndrome characterized by transient fever and catarrhal changes in the ocular, buccal, and naso-pharyngeal mucosae. Blood from reacting dogs inoculated into other dogs engendered similar symptoms. More than 50 passages were carried out in the period 1936-38 but they were abandoned because of the onset of the Second World War. The only experimental tool for measles isolation available at that time was the monkey and monkeys were difficult to procure. The author, however, claimed that the infective agent in his dogs was destroyed by heating at 56°C for 30 minutes and by treatment with formaldehyde. It was neutralized by human measles convalescent serum but not by Carre's disease hyperimmune serum. Rjazantseva (1956) whom Goret (1961) cited in support of his findings, induced a febrile syndrome in pups with measles virus but unfortunately he sought to establish the identity of the virus in his pups by an almost unheard of technique which he called "agglutination of bacteria-bearing virus".

RINDERPEST

Natural infections: Rinderpest has a wider natural and experimental host range than the other two viruses. The most important natural hosts are cattle and water

buffaloes. Goats and sheep are susceptible, but outbreaks in these species are rare. In the past decade only three reports of the natural disease in goats are authenticated (Johnson, 1958; Libeaux and Scott, 1960; Sharma, 1965). On the other hand, recent incontrovertible serological evidence indicated that past infection had occurred in sheep and goats in West Africa (Zwart and Rowe, 1966). It remains to be seen, whether this is the forewarning of the emergence of a new stable complex of the disease in small ruminants or whether it is "peste des petits ruminants", a natural disease of small West African ruminants that simulates rinderpest clinically and pathologically (Gargadennec and Lalanne, 1942; Mornet, Orue and Gilbert, 1956a).

The aetiological agent of "peste des petits ruminants" has been characterized as a myxovirus morphologically and cytopathogenetically and immunologically related to bovine rinderpest (Mornet, Orue, Gilbert, Thiery and Mamadou, 1956b; Gilbert and Monnier, 1962a). "Peste des petits ruminants" is perhaps an extreme form of adaptation of rinderpest to sheep and goats. The virus does not appear to spread from infected sheep and goats to cattle (Monnet et al., 1956b) but inoculation of the virus into cattle conferred some resistance to rinderpest without the development of appreciable clinical symptoms (Gilbert and Monnier, 1962b).

Natural infection probably occurs in camels but clinical reports have not been confirmed virologically. Experimentally, camels have been infected (Tartakowsky, 1899; Lingard, 1905; Curasson, 1932, 1942) and recently, Taylor (1968) reported spread of the experimental disease from cattle to camels kept in close contact.

Rinderpest attacks the Asian domestic pig under natural circumstances. Outbreaks in Indo-China (Carre and Frainbault, 1898) and on the Rombolon Island of the Philippines have been discussed by Scott (1964) and Plowright (1965, 1968). Elsewhere, the pig probably played a role in the initiation of the virgin epizootic in Western Australia in 1923 (Robertson, 1924) and pigs were suspected as having spread the virus in Belgium in 1920 (Nicolas and Rinjard, 1921). The disease in the Asiatic pig is overt but in pigs of European origin the infection is mild or sub-clinical (Molinie, 1931; Scott, DeTray and White, 1959; Scott et al., 1962). Pigs can acquire infection by ingesting infected scraps of meat and there is, therefore, always a risk of introducing rinderpest to a previously disease-free country through the importation of carcases and offal from enzootic countries (Scott et al., 1962).

Scott (1959, 1964) has documented authoritatively the evidence on the susceptibility of free-living and captive wild artiodactyls. He also furnished a

comprehensive list of species in which rinderpest infections have been alleged but not proven.

Experimental infections: The experimental adaptation of bovine strains of rinderpest virus to goats (Edwards, 1930; Stirling, 1932, 1933; Saunders and Ayyar, 1936; Daubney, 1948, 1949), rabbits (Inoue, 1934; Cebe and Perrin, 1935; Nakamura, Wagatuma and Fukusho, 1940; Cheng and Fischman, 1949) and fowl embryos (Shope, Griffith and Jenkins, 1946) was a notable achievement in the history of rinderpest control because stable mutants obtained from the experimental hosts were used as live attenuated virus vaccines. The virus caused discernible pathological effects in all these species but the lesions were pathognomonic only in rabbits.

Rinderpest virus has also been propagated in susliks, Citellus mongolicus, (Inoue, Haroda and Shimizu, 1930) in white mice and golden hamsters (Scott and Whitcomb, 1958) and in European types of pigs (Carmichael, 1940). Propagation has been claimed in guinea-pigs (Inoue, 1934), giant rats, Cricetomys gambianus, (Curasson, 1932) and day-old and very young chicks (Baker and Greig, 1946; Shope and Griffiths, 1940). More recently, the virus was successfully adapted to the brain of sucking mice (Imagawa, 1965).

Consideration of the behaviour of rinderpest virus in dogs and ferrets is relevant in the context of the demonstration of its close relationship to the virus of

canine distemper. Earlier attempts to passage rinderpest virus in dogs yielded negative or equivocal results (Hallen, Macleod, Charles, Kerr and Jan, 1871; Curasson, 1932$_a$). Viseur (1771) fed rinderpest infected meat to dogs and recovered the virus by feeding their faeces to susceptible calves. Morcos (1931) observed mild fever in dogs inoculated with but not fed rinderpest virus. A single susceptible bull receiving blood collected after three passages in dogs showed no clinical signs but it resisted challenge with rinderpest virus; the experiment did not include controls.

The first recorded serological evidence of rinderpest neutralizing antibodies in dogs stems from the work of Polding and Simpson (1957). Their findings were confirmed later by Scott and Brown (1958). Puppies inoculated with rinderpest virus developed sub-clinical disease associated with the presence of virus in blood which proved to be infective in a susceptible ox (Polding et al., 1959).

There is no proof of transmission of rinderpest virus to ferrets. Inoculated ferrets remained healthy and did not develop rinderpest antibodies (Mornet, Goret, Gilbert and Goueffon, 1960).

CANINE DISTEMPER

Natural infections: The natural spectrum of infectivity of canine distemper virus is restricted to four families

of the Order Carnivora, namely, Canidae, Mustelidae, Procyonidae and Viverridae. Most reports of distemper in wild carnivores are based on clinical observations in zoological gardens (Armstrong and Anthony, 1942; Goss, 1948).

Laosson (1882) was the first to report on the natural disease in foxes. Later, Dalling (1933) and Green and Carlson (1945) described similarities in the natural disease of the fox and the domestic dog. Likewise, Pinkerton (1940) reported similarities in the clinical and histological features of fox and mink distemper.

The racoon, Procyon roctor, and the binturong, Arctictis binturong, are the only members of the Procyonidae and Viverridae respectively, known to have been affected in the wild state (Helmboldt and Jungherr, 1955; Kilham, Habamann and Herman, 1956). The virus has been allegedly isolated from captive pumas, Puma concolor, and captive tigers, Panthera tigris (Harispe and Waine**y**, 1941) and captive lions, Panthera leo, (Piat, 1950).

Zuill (1895) mentioned that hyenas, species unnamed, were susceptible. Scott (1964) and, more recently, Taylor (1966) found antigens and antibodies in the tissues of spotted hyenas, Crocuta crocuta, reactive with both rinderpest antigens and antibodies. Another alleged natural host is the American badger, Taxidea taxus,

(Armstrong, 1942; Farrell, 1957).

Reports of subclinical infection in man (Bryan, 1928; Nicolle, 1931; Vuori, 1946; Kirk, 1941; Adams, 1953; Goret, 1961) and monkeys (Zuill, 1895; Martin, 1950; Schwarz, York and Pitman-More, 1960; DeLay et al., 1965) are not supported by virological data. Mere demonstration of antibodies to distemper in man is not evidence of past contact with canine distemper because infection with measles virus produces antibodies reactive with canine distemper virus (Karzon, 1955).

Experimental infections: Rabbits are generally regarded as refractory to canine distemper infection (Gilbert et al., 1960). The only rodents to which the virus has been successfully adapted are sucking mice (Morse, Chow and Brandly, 1953; Gutierrez and Gorham, 1955; Kurata, Kaizuka and Fujie, 1963) and sucking hamsters (Waksman et al., 1962).

The experimental host most exploited in distemper research is the fowl embryo. Propagation of wild strains of the virus resulted in progressive attenuation of virulence for dogs and ferrets (Plummer, 1938; Haig, 1948, 1949; Cabasso and Cox, 1949). Further, unlike growth of measles and rinderpest viruses, the growth of canine distemper virus in embryonated hen eggs results in pathognomonic lesions that permit detection of virus activity and facilitate the titration of distemper antibodies.

PATHOGENESIS

In general, experimental data on the pathogenesis of the three diseases are in harmony with Fenner's concept of virus pathogenesis as exemplified in ectromelia infections of mice (Fenner, 1948). There is, however, no evidence of their primary replication in the epithelial cells of the respiratory mucosa.

Following intranasal instillation rinderpest virus was demonstrable 24 to 48 hours later in the pharyngeal and mandibular lymph nodes as well as in the palatine tonsil. Viraemia occurred one to two days after the appearance of the virus in the lymph nodes. (Leiss and Plowright, 1964; Taylor, Plowright, Pillinger, Rampton and Staple, 1965). Using the fluorescent antibody technique, Liu and Coffin (1957) demonstrated distemper virus antigens in the phagocytic cells of respiratory mucosa and in increased amounts in the reticular cells of the cervical lymph nodes two days after intranasal inoculation of the virus. Thereafter the antigen was successively traced in the mediastinal and mesenteric lymph nodes, spleen, Kupffer cells of the liver, and in the leucocytes of peripheral blood. In other words, spread was lympho-haematogenous and ended in viraemia. Antigen did not appear in the nasal and bronchial mucosa until the seventh day after exposure (Liu and Coffin, 1957).

Following replication of the regional lymph nodes the viruses of measles, rinderpest and distemper invade the blood. Primary viraemia is restricted and transient and further replicative events occur in lympho-reticular organs and tissues resulting in a readily demonstrable viraemia which precedes pyrexia. Virological evidence for this type of dissemination occurs in the work of Plowright (1964a) and Taylor (1965) on rinderpest and in the detailed quantitative studies of Crook, Gorham and McNutt (1958) and Crook and McNutt (1959) on mink and ferret distemper. The evidence for the dissemination of measles virus, however, is clinical and histological. Generalized lymphadenopathy and splenomegaly occurring six to eight days after natural exposure indicates involvement of the reticulo endothelial and lymphoid organs (Herman, 1914; Friedman, 1927, 1931).

Viraemia in all three infections lasts until the tenth to fifteenth day after the [illegible] but it can be protracted in distemper (Rockborn, 1958; Cornwell, Campbell, Vantsis and Penny, 1965a, 1965b). The virus is readily demonstrable in or cultivable from the leucocytes (Gresser and Chany, 1963; Plowright and Ferris, 1962b; Tokuda, Fukusho, Morimoto and Watanabe, 1962, 1963; Rockborn, 1958b). Viraemia and virus replication in the lympho-reticular organs entail dissemination of the virus in the target organs, the damage to which engenders the characteristic prodromata.

The decline of viraemia generally coincides with the appearance of humoral antibodies. These have been detected in measles one to three days after the rash (Ruckle and Roger, 1957), in rinderpest 6 to 21 days after exposure (Plowright and Ferris, 1962b) and in canine and mink distemper six days after exposure (Rockborn, 1957; Gillespie et al., 1958). In the terminal stages of viraemia and also in the protracted viraemia that frequently occurs in distemper, virus and antibody often co-exist, the virus being intracellularly located in the leucocytes (Gorham, 1960; Plowright and Ferris, 1962a; Gresser and Chany, 1963). Gorham (1960) described an outbreak of distemper in ferrets following injection of virus-contaminated anti-serum. Rockborn (1957a) observed in cases of prolonged viraemia that died that the rise in neutralizing antibodies was slow. Ott et al. (1955), likewise, found low levels of antibodies in the later stages of severe, protracted distemper. They expressed the view that prolonged viraemia was responsible for antibody suppression but did not provide virological evidence.

In measles it is difficult to correlate the disappearance of viraemia or clinical symptoms with the emergence of circulating antibodies. Passively administered gammaglobulin can ameliorate clinical symptoms and the [illegible] injection of antibodies can prevent the appearance of the rash (Debre, Bonnet and

Broca, 1923). On the other hand, most patients with agammaglobulinaemia recover from measles when infected (Janeway and Gilin, 1957). Significantly, agammaglobulinaemic patients have interferon in their leucocytes while infected with measles (Gresser, 1961) and it is tempting to speculate that recovery from measles, rinderpest and distemper is associated with interferon production whereas the duration of immunity is related to neutralizing antibodies. Measles and rinderpest viruses are known to stimulate the production of interferon in vitro (DeMaeyer and Enders, 1961; Plowright and Finter cited by Plowright, 1968). Moreover, attenuated strains of measles virus induce higher quantities of interferon than virulent strains (DeMaeyer and Enders, 1965).

Petralli, Merigun and Wilbur (1965) compared the levels of interferon and neutralizing antibodies in the sera of children given live attenuated measles vaccine. Interferon was detected during the fever 7 to 11 days after vaccination in 95 per cent, the highest levels being obtained on the ninth or tenth day. Antibodies were detected on the eleventh day (Stokes, Reilly, Hilleman and Buymak, 1960). The results confirmed earlier clinical observations on the efficacy of live measles vaccine in modifying the natural disease if administered within three days of exposure (Berkovich and Starr, 1963; Brody and Bridenbaugh, 1964).

Similar amelioration of natural rinderpest infections in cattle by injecting attenuated virus were reported in the late 1930's (Pfaff, 1938; Wilde and Scott, 1961).

Recovery from measles, rinderpest and distemper does not lead to a chronic carrier state but results in a substantial immunity. Rinderpest virus inoculated into immune animals generally disappears immediately (Scott, 1955). Recently, however, Plowright and Taylor (1957) observed transient viraemia without clinical reactions in two successfully vaccinated animals which were challenged with virulent virus. Likewise, it has been demonstrated that monkeys immunized with live measles vaccine can be reinfected (Enders, Katz and McLearis, 1959). There was local multiplication and no viraemia. Warren, Kammer and Gallian (1962), on the other hand, detected virus in blood despite the presence of circulating antibodies. Nevertheless, reinfection resulted in an accelerated antibody response. The conclusion that reinfection can occur in measles, rinderpest and distemper, therefore, seems inescapable. Presumably, the extent of virus replication in reinfection depends on the level of antibody present and on the speed of the anamnestic response.

PATHOGENICITY

The cytopathologies of measles, rinderpest and canine distemper viruses have features reminiscent of those produced by the parainfluenza viruses. Growth in cell culture is accompanied by the formation of multinucleate cells or syncytia containing cytoplasmic and nuclear inclusion bodies. The former appear earlier than and out number the latter. The frequency and rapidity of occurrence of syncytia and the inclusions depends upon a multiplicity of factors such as the strain of virus, its passage history, the concentration of the inoculum, the type of cell culture, the state of metabolic activity of the cells, the composition of the cell culture medium and the temperature of incubation.

HISTOPATHOLOGY

The first hint of similarities between measles, rinderpest and distemper emerged from histological findings. The three viruses have a striking affinity for the cells of the lympho-reticular system. Vasculo-endothelial proliferation and necrosis and lympho-cytolysis are the principal features in the histo-pathology of the diseases they produce in their natural hosts.

Mesenchymal giant cells have been demonstrated in the lympho-reticular tissues of the throat, respiratory

and alimentary tracts, and in the spleen and lymph nodes during the prodromal phase of measles (Herzberg, 1932; Feyrter, 1947) and their occurrence in nasal secretions provides a presumptive diagnostic test (Tompkins and Macaulay, 1955). Sometimes mesenchymal giant cells develop during the incubation period. Warthin (1931), Finkeldey (1931), Hathaway (1935) and Sherman and Ruckle (1958) described them as large spherical or irregular cells with variable and often numerous nuclei distributed in a cluster in the centre. They generally disappear before the rash is obvious (Feyrter, 1947) but may persist into the early rash period (Stryker, 1940; Corbett, 1945).

Giant cell infiltration is pathognomonic for natural and experimental measles. Monkeys inoculated with the virus develop giant cells in their lymph nodes and spleen between the third and seventh days of infection. Their appearance is generally accompanied by exhaustion of the germinal centres and by reticulo-endothelial hyperplasia, (Gordon and Knighton, 1941), a picture reminiscent of that in natural bovine rinderpest (Smith and Jones, 1961).

In rinderpest infections multinucleated cells appear in the lymphoid tissues and spleen on or about eighth day after infection (Khera, 1958a) and the presence of eosinophilic, cytoplasmic inclusion bodies together with the pattern of nuclear degenerative

changes are regarded as specific (Thiery, 1956). Similar cytological changes were observed in small ruminants infected with "Peste des petits ruminants" (Mornet et al., 1956b). Plowright (1962a) noted that cytoplasmic inclusions were numerous but nuclear inclusions rare in the cases of rinderpest examined by him.

The giant cell reaction in canine distemper is limited to the lungs and the lymphoid tissue (Jennings, 1967). It was originally described and depicted in distemper of mink, ferrets and foxes by Pinkerton et al., (1945) who also observed cytoplasmic inclusions in 50 per cent of the giant cells; nuclear inclusions were rare.

In rinderpest also the epithelium of the labial, buccal, pharyngeal, vulvar and preputial mucosae as well as the skin of the lips show evidence of syncytium formation (Khera, 1958$_b$). The initial changes are characterized by hypertrophy and hyperplasia and ballooning degeneration in the cells of the Stratum spinosum which later enlarges and extends to form distinct foci of acanthosis and spongiosis. Acidophilic cytoplasmic and nuclear inclusions occur in the foci of ballooned cells (Khera, 1958$_b$). Whether or not these inclusions are virus-induced can only be substantiated by electron microscopic and fluorescent-antibody studies but there is no such report to date.

CYTOPATHOLOGY

The progression of cytopathic changes in human and simian cells infected with measles virus was first depicted by Enders and Peebles (1954). Specific and reproducible changes associated with the replication of rinderpest virus in cell cultures were first demonstrated by Plowright and Ferris (1957). A year later, Rockborn (1958a) provided unequivocal proof that canine distemper virus grew in primary canine renal epithelial cells and that the cytopathogenetic effects were sequential, specific and regularly reproducible.

Growth of virulent strains of measles virus in primary cultures was generally slower than the growth of adapted strains either in primary cultures or in cell lines. (Matumoto, 1966; Norrby, 1967). On the other hand, growth in virulent strains of rinderpest virus in bovine cell cultures was relatively rapid; titres of 10^3 to 10^4 TCD_{50} per ml were obtained between the second and fourth days (Plowright, 1968). Growth of distemper virus in canine cells was much slower and infectivity titres were usually one or two log units less than those of measles and rinderpest viruses grown in homologous cultures (Rockborn, 1958a; Bittle et al., 1961).

Although the range of cytopathogenicity of measles, rinderpest and distemper was varied, the evolution of cytopathic changes in the cells infected by the three viruses was essentially similar. The earliest change

was the appearance in the cell sheet of discrete foci of varying size and shape in which the cell boundaries had disappeared and the nuclei were difficult to visualize. The foci were referred to as "glassy" plaques. (Enders and Peebles, 1954). Pari passu small and large vacuoles developed in such foci. In stained preparations, the plaque-like structures with vacuoles were recognized as small groups of multinucleate-cells or syncytia containing cytoplasmic and nuclear inclusions (Enders and Peebles, 1954; Plowright and Ferris, 1957; Bittle et al., 1961).

The time of onset of the changes was, however, different in respect of the three viruses. In cultures infected with measles and rinderpest viruses, syncytia were manifest on the fourth or fifth day. In distemper, Bittle et al. (1961) detected them on the eighteenth day after seeding. Rockborn (1958_a), however, first spotted cytopathic changes 42 days after infection. Vantsis (1959) reported wide variations in the onset of cytopathic changes. Sometimes syncytia were detected only after five weeks. At other times they occurred on the third or fourth day. In direct cultures of infected organs cytopathic changes were evident on the sixth or seventh day.

Time-lapse microcinematography of measles-infected cultures revealed two types of giant cells, the small and large (Thomison, 1962). The small had 4 to 10

nuclei, rarely up to 25 nuclei, and a dense cytoplasm which had filamentous borders. There was considerable streaming of the cytoplasm and the nuclei were much smaller than those of the adjacent cells. The small syncytium generally showed restricted movement. The large giant-cell appeared first as the product of fusion of 2 or 3 cells. Its growth increased in 24 to 48 hours by incorporation of adjacent cells. Cytoplasmic movement was marked but nuclear movement was restricted and sharply contrasted with the marked movement of nuclei throughout the expanse of cytoplasm that was described in Herpes simplex infections by Barski and Robineaux (cited by Thomison, 1962).

In rinderpest, a high input multiplicity of infection resulted in the appearance of large numbers of small syncytia on the third or fourth day. They contained several nuclei and reached their maximum size between the sixth and tenth days. A second cycle of syncytial formation developed reaching a peak about the fourteenth to eighteenth day and consisting of much larger masses derived apparently from fusion of the small, less-vacuolated syncytia. Maximum infectivity titres were obtained at this stage (Plowright, 1968). Gilbert and Monnier (1962a, 1962b) described similar sequential changes in cultures infected with rinderpest and "peste des petits ruminants" viruses. They reported the large cyncytial masses developed better at 40°C than

at 37°C.

Rapp (1960) thought that the presence of small and large syncytia in measles cultures reflected differences in generation times. Toyoshima et al. (1969a) expressed the view that they represented different modes of infection; the small syncytium was perhaps the product of fusion of simultaneously infected cells whereas the large syncytium developed in a progressive fashion over a period of 24 to 36 hours. They found no antigen in the cells in the first 10 hours. Similar cells were produced with virus irradicated with ultraviolet light. It was, therefore, concluded that the large syncytia resulted from multiple infection and was not caused by the spread of virus from cell to cell. It is difficult to reconcile this hypothesis with the finding of Rapp, Gordon and Baker (1960) that measles antigen diffused professively from cell to cell in cultures maintained in the presence of an excess of measles neutralizing antibody unless it is presumed that uninfected normal cells are also incorporated into the syncytium in the course of its evolution. Such a possibility was mooted by Ayoma (1959).

The small, stellate syncytia were produced more frequently by freshly isolated strains of rinderpest virus of low virulence (Plowright, 1962a, 1963a; Leiss, 1963). On an analogy with the observations of Roizman and Schluederberg (1962) that the small, stellate type

syncytia in measles-infected Hep-2 cultures had probably arisen from the cells infected while in mitosis, Plowright (1968) considered that the small syncytia in rinderpest cell cultures had a similar origin.

The syncytium in distemper cultures was indistinquishable from the syncytium in measles and rinderpest cultures. A variety of patterns in the giant-cells has been discussed by Rockborn (1958a) and Shaver et al. (1964).

In his comparative study of the cytopathology of measles and distemper Shaver et al. (1964) observed that ferret kidney cultures inoculated with distemper virus, syncytia were prominent on the fourth day. Their numbers increased gradually up to the thirteenth day and then declined steadily. Likewise, in measles cultures syncytia were evident on the fourth day and gradually increased in size and numbers through the ninth day (Shaver et al., 1964). The so-called "contracted multinucleate cells" observed by Shaver and his colleagues in measles and distemper cultures represented, according to them, a degenerate type of syncytium containing several hyperchromatic and pyknotic nuclei.

Measles, rinderpest and distemper syncytia have a characteristic cytomorphology. The typical syncytium had a broad expanse of cytoplasm which was granular and vacuolated and contained numerous nuclei randomly scattered or, more usually, arranged in a cluster. They

arose from cell fusion (Bech and Von Magnus, 1958; Rockborn, 1958a; McCarthy, 1959; Thomison, 1962).

A notable cytological hallmark of measles, rinderpest and distemper cytopathologies was the appearance of cytoplasmic and nuclear inclusions. The former were generally numerous and appeared early, particularly with high multiplicity infection. They occurred in single cells and in syncytia. The intranuclear inclusions occurred much later but were generally scanty. In primary cells seeded with wild strains of measles, rinderpest, "peste des petits ruminants" and distemper viruses they were more prominent (Enders and Peebles, 1954; Plowright and Ferris, 1959a; Bittle et al., 1961; Plowright, 1962a; Gilbert and Monnier, 1962a; Rao, 1963). With serial passage they became less frequent and were not demonstrable in certain cell systems such as Girardi heart cells (Black et al., 1959), fowl embryo cells (Katz et al., 1958), LU 106 cells (Norrby et al., 1964a), Bsc-1 cells (Johnson and Ritchie, 1969). In their studies with wild strains of canine distemper virus, Rockborn (1958a) and Hopper (1959) did not detect inclusion bodies in primary dog and ferret tissue cultures.

Several workers have sought a morphological correlation between the inclusion bodies and the accumulation of virus antigens detectable by histochemical stains (Toyoshima et al., 1960; Leiss, 1964; Provost,

Borredon and Queval, 1965), fluorescent antibody techniques (Cohen et al., 1955; Toyoshima et al., 1960a; Appel and Jones, 1967) and electron microscopy (Breese and De Boer, 1963; Provost et al., 1965; Tajima, Ushijima, Kishi and Nakamura, 1967; Nakai, Shand and Howatson, 1969).

In measles cultures although antigens were first traced in the perinuclear zone of the cytoplasm and later in the periphery, no intranuclear antigen was evident, except occasionally, at a later stage of infection (Cohen et al., 1955; Rapp et al., 1960). In certain cell systems, however, intranuclear fluorescence was evident (Roizman and Schluederberg, 1962). In measles cultures stained by acridine orange, Toyoshima et al. (1960a) failed to find any morphological link between nucleic acid and the inclusion bodies. Such a link was, however, established in rinderpest cultures by Leiss (1964) and Provost et al. (1965). Very early in their development, the cytoplasmic inclusions emitted orange fluorescence when stained with acridine orange. The structures associated with the effect were identified as cytoplasmic inclusions by light microscope (Leiss, 1964). In ultrathin sections, they were found to be made up of collections of enlarged and distorted ergastoplasmic vesicles, mitochondria, and nucleocapsids (Provost et al., 1965). Sometimes, numerous polyribosomes gathered around the ergastoplasmic vesicles which con-

tained particles 500-700 angstroms in diameter. Recently these findings were confirmed and extended by Tajima et al. (1967) and Nakai et al. (1969) in cultures infected with rinderpest and measles viruses respectively. On the other hand, the nature and specificity of the inclusion bodies in distemper-infected cultures have not been studies in detail.

HAEMAGGLUTINATION

Measles, rinderpest and distemper cultures contain, besides infective virions, a heterogeneous group of particles, which are composed of virus envelopes in one form or another. Rinderpest and distemper cultures contain antigenic fractions which participate in immunodiffusion and complement-fixation reactions (Bindrich, 1954; Mansi, 1955; White, 1958a, 1958b; Scott and Brown, 1961; Ishii, Tokuda and Watanabe, 1964). Measles cultures, however, show an array of biological activities such as haemagglutination, haemadsorption, haemolysis, fusion-inducing and complement-fixing properties. The particles associated with these activities can be separated from intact virions by physical methods using rate zonal and equilibrium centrifugation in sucrose and caesium chloride density gradients respectively (Schluederberg and Roizman, 1962; Norrby, 1963; Norrby et al., 1964a).

HAEMAGGLUTINATION BY MEASLES VIRUS

Physico-chemical aspects: The term haemagglutinin has been applied to different forms and components of the virus, all of which exhibit haemagglutinating activity. By rate zonal centrifugation, Norrby (1963) separated two major fractions, the rapidly sedimenting, the so-called "large haemagglutinin" and the more slowly sedimenting,

the "native small haemagglutinin".

The "large haemagglutinin" was relatively homogeneous in caesium chloride density gradients. Part of the fraction having a density of 1.24 to 1.25 grams per ml carried the infectivity. This density is different from the 1.29 grams per ml for the infectious particle quoted by Schluederberg and Roizman (1962). The reason for the discrepancy is obscure. The "large haemagglutinin" also carried the bulk of haemolytic and complement-fixing activities and the viral RNA and only 10 per cent of the total haemagglutinating activity.

The "native small haemagglutinin" was more heterogeneous than the "large haemagglutinin" and contained 90 per cent of the total haemagglutinating activity. Three main groups of particles were recognised; low density particles with densities ranging from 1.13 to 1.21 grams per ml, a group with densities ranging from 1.13 to 1.25 grams per ml, and higher density particles of 1.29 to 1.30 grams per ml. The low density particles exhibited both haemolytic and haemagglutinating activities. Norrby (1963) thought that they represented complete, almost complete, and empty envelopes.

The second group only possessed haemolytic activity. Most of the particles were disintegration products of the virus envelope but, in addition, some resembled the empty virions described for Newcastle disease virus by Rott, Reda and Schaffer (1962) and

Schluederberg and Roizman (1962) suggested that they represented denatured or "incomplete" virus. Waterson (1965) however, stressed the fact that the particles were not analogous to the multiplicity-dependent incomplete forms of influenza virus and that there was no evidence that the von Magnus phenomenon occurred with measles virus.

The third group of particles in Norrby's "small haemagglutinin" fraction only possessed haemagglutination activity. Their higher density was attributed to a low lipid content.

Conversion of the "large haemagglutinin" into "small haemagglutinin" was effected by ether and more consistently with Tween 80-ether treatment (Norrby, 1962b, 1962c; Waterson, Rott and Ruckle-Enders, 1963). The effect of treatment with ether on human influenza virus was first described by Hoyle (1950). The virus was split into two fractions, the haemagglutinin and the nucleoprotein. Similar results were obtained by Schaffer and Rott (1959) and Sokol, Blaskovic and Rosenberg (1961), with Sendai, Newcastle disease and mumps viruses. To reduce the denaturing effect of ether, Hosaka, Hosakawa and Fukai (1959) incorporated a surface-active reagent, Emasol, 1130, during ether treatment of Sendai virus. This combination of detergent and ether was later successfully applied to measles virus suspensions (Norrby, 1962b, 1963, 1964b;

Funahashi and Kitawaki, 1963; Waterson et al., 1963; Kitawaki, Funahashi and Toyoshima, 1964). Tween 80 was substituted for Emasol as the latter was slightly haemolytic. It was found that while treatment with ether per se caused no change, or sometimes, reduction of titres, Tween 80-ether treatment caused a 4 to 32-fold increase in the haemagglutination titre (Norrby, 1962b, 1963c; Peries and Chany, 1962).

The sequences in the splitting of the "large haemagglutin" fraction by Tween 80-ether treatment have been investigated by Norrby (1964b) using caesium chloride density gradients. Untreated material showed two bands, a sharp band at a density range of 1.29 to 1.30 grams per ml which exhibited only haemagglutinating activity, and a broader band extending from 1.13 to 1.25 grams per ml. Infectivity was demonstrable in the high density part of the broad band whereas haemolytic and complement-fixing activities were maximal at 1.23 to 1.24 grams per ml. The two activities, however, appeared to be distributed throughout the broad band, albeit there were small differences.

Tween 80-ether treatment caused a complete disappearance of the broad band both visually and in biological activity assays. Only the small, sharp band remained and it exhibited a markedly enhanced haemagglutinating activity. It also showed some complement-fixing activity.

The readiness with which the material in the sharp bend was adsorbed on to monkey erythrocytes engendered the hypothesis that either the dissociated envelope materials had complement-fixing activity or some of the nucleocapsid was still bound to the fragmented envelope. Electron microscopic examination however, supported the second possibility.

Density gradient studies revealed the presence of a third fraction with a much higher density, namely, 1.32 grams per ml (Norrby, 1964b). The fraction showed only complement-fixing activity which was not adsorbed out by erythrocytes. These findings together with electron microscopic examinations provided the proof that complement-fixing activity was associated with the nucleocapsid material which, before Tween-ether treatment was "immured inside the intact virion" (Waterson, 1965). Tween-ether treatment, therefore, appears to convert all the envelope material, be it the intact virion, dissociated virion, and the "empty" intact forms containing no nucleocapsid to smaller more uniform particles. Electron microscopy revealed that the small particles were rosette-like (Norrby, 1964a; Waterson, 1965) and similar to those described for Sendai virus by Hosaka, Nishi and Fukai (1961) and for Newcastle disease virus by Rott and Schaffer (1961). On the other hand, rate zonal centrifugation in sucrose density gradients revealed marked heterogenicity in respect of sedimentation

character (Norrby, 1966).

Several other detergents and organic solvents have been employed to disintegrate measles virus particles. Waterson et al. (1961) used Triton X-100 with success. Kanarek (1964) employed tetrachlorethylene and fluorocarbon in combination with Tween 80 and found that the disruption was efficient and did not entail loss of immunogenicity. Following the reported success of deoxycholate treatment for influenza virus by Laver (1963), Norrby (1966) examined the effectiveness of the method in the fractionation of measles haemagglutinins. About a 100-fold increase in haemagglutination titre was obtained using deoxycholate in a final concentration of 0.05 per cent. The material obtained was labile which precluded its use in measles haemagglutination-inhibition tests. Nevertheless, Norrby (1966) recommended the procedure for separating haemagglutinins from nucleocapsid structures.

A salt-dependent agglutinin has been recently isolated from measles-infected cells. It adsorbed on to monkey erythrocytes only in hypertonic salines (Schluederberg and Nakamura, 1967) and it seems to be closely related chemically to the Tween 80-ether split haemagglutin. It sedimented with a coefficient of 22S and had a buoyant density in caesium chloride of 1.30 grams per ml. The salt-dependent agglutinin is probably a protein with characteristics similar to those of the

slow-sedimenting haemagglutinins in measles cultures and may represent a precursor of the measles envelope proteins (Schluederberg and Nakamura, 1967).

Biological Properties: Measles haemagglutination was observed independently by Mastyukova and Khait (1960) and Peries and Chany (1960). The former gave more details of haemadsorption of rhesus monkey erythrocytes in measles-infected cultures than haemagglutination. Culture fluids had haemagglutinating activity in a dilution of 1:4. Peries and Chany (1960, 1961), on the other hand, used concentrated tissue culture fluids and demonstrated haemagglutination and partial haemolysis of baboon (Papio ursinus) and Patas monkey (Erythrocebus patas) erythrocytes. Their results were quickly confirmed (Albano, 1961; De Meio and Gower, 1961; Rosanoff, 1961; Rosen, 1961; Black, 1962).

Production: With few exceptions, the titre of haemagglutinins obtained in most culture systems is generally poor. The release of haemagglutinins seems to be determined by factors other than the amount of infectious virus present in a preparation. In general, the ratio of infectivity titre to haemagglutinin titre ranged from 10^3 to 10^5 (Bussell and Karzon, 1966). Primary simian, human and canine cultures have often yielded higher titres of haemagglutinins than cell lines notwithstanding the fact that the rate of growth and infectivity titres obtained were much higher in the

latter (Norrby, 1962a; Ruckle-Enders, 1962, 1964).

Tween-ether treatment of crude virus suspensions from primary cultures gave higher titres than the titres from cell line suspensions similarly treated (Norrby, 1967). The explanation lies in the observation that during active virus multiplication an equilibrium between release and thermal decay of infectious virus particles is established. Consequently, the rate of virus replication and the time allowed for the accumulation of non-infectious antigens decides the relative ratio of the two in the material harvested from time to time (Black, 1959a; Norrby, 1962a, 1964a; Togo, 1964). The behaviour of adapted strains of measles virus in fowl embryo cultures is, however, an exception to the generalization. Haemagglutinins have never been demonstrated in this system but the infectivity titres are generally much lower than in mammalian cell lines (Rosanoff, 1961).

The observation of Ruckle-Enders (1962) that fresh isolates of measles virus failed to produce haemagglutinin gave rise to the speculation that haemagglutinin production was genetically determined. However, on serial passage of non-agglutinating strains in primary cultures haemagglutinins did appear and their appearance coincided with the change from giant-cell to spindle-cell degeneration (Bussell and Karzon, 1966). It could be argued that the appearance of the haem-

agglutinin had stemmed from increased infectivity titres that had occurred on serial passage. However, subsequent observations by Waterson et al. (1963) that Tween-ether treatment failed to release haemagglutinins in cultures of the giant cell variants has strengthened the hypothesis that haemagglutinin production and cytopathic strand formation are genetically determined.

Reports on the kinetics of haemagglutinin production in measles cultures are scanty. Norrby (1962a) studied the relationship between infectivity and haemagglutinin titres in measles infected human and dog renal cells maintained at 30, 33 and 37°C. Haemagglutinins were first detected on the eighth day at 37°C and 33°C and on the eleventh day at 30°C. Thereafter, the rate of production was nearly the same at all three temperatures and the final titres were reached in 16 to 29 days. The ratio, $TCID_{50}$/HAU, varied between 10^3 and 10^4 at 33°C which was the optimal temperature for the production of infectious particles. The ratio increased as long as the cells continued to release virus. After that there was a sharp decrease, presumably caused by the thermolability of the infectious particles. At 37°C and 30°C there was no significant shift in the ratios except for the terminal decline. To ensure maximal haemagglutinin titres, Norrby (1962a) maintained some cultures in bovine amniotic fluid which was not renewed after the appearance of the first cytopathogenic

effect. Titres of up to 512 HAU per 0.4 ml of the medium were obtained. Togo (1964) noted maximal haemagglutinin titres in Hep-2 cells 10 to 12 days after infection with high dosage levels; the titres were far lower than those obtained by Norrby (1962a).

Using an input multiplicity of approximately one plaque-forming unit per cell, Numazaki and Karzon (1966) followed the appearance of biological activities in AV_3 cells infected with the Edmonston strain of measles virus. Cytopathic effects were first noted at 12 hours after infection and were manifested by the appearance of a few small syncytia. By 24 hours more than 90 per cent of the cells had fused. The first increase in cell-associated infectivity occurred at 12 hours after infection and proceeded logorithmically up to 24 hours. Cell-associated complement-fixing activity was demonstrated at 16 hours after infection and cell-associated haemagglutinin and haemolysin were detected 3 hours later. All the three activities increased until the 48th hour. In the released fraction, infectivity and complement-fixing activity were demonstrable at 24 and 48 hours and haemagglutinin and haemolysin were detected at 70 hours.

Cell-disruption procedures such as freezing and thawing and sonication have been used to release cell-bound haemagglutinins and other antigens. Numazaki and Karzon (1966) found that sonication was more effective

than freezing and thawing and caused a four-fold increase in the yield of haemagglutin.

Schluederberg (1968) reported significant enhancement of haemagglutination titres of unfractionated virus pools by salts such as ammonium sulphate. The titre increase depended upon the ratio of "enhanceable" haemagglutinins to "non-enhanceable" haemagglutinins in a preparation. Sonication converted the "non-enhanceable" particles into the "enhanceable" state. She postulated that salt-enhanceability may be a function of the membrane integrity. In other words, envelope-integrated haemagglutinins do not show increase in activity in the presence of the salt. The haemagglutinins are "non-enhanceable". Haemagglutinins that are not integrated into the viral envelope are salt-dependent for their activity while envelopes that have been modified physically by sonication may have intermediate properties (Schluederberg, 1968).

Red cell spectrum: Measles virus agglutinates only simian erythrocytes. Apparently, there are small differences in the sensitivities of red cell suspensions from different species of monkeys and the general opinion is that the erythrocytes of the African Grivet monkey (Cercopithecus aethiops) are the most sensitive (Ruckle-Enders, 1962; Cutchins, 1962; Norrby, 1963). According to Black (1962) Berkley Rich made a detailed study of the subject and found that cells from

anthropoids and prosimians were not agglutinated. Cells from Potto (Perodicticus potto), Galago (Galago crossicandatus), and Lemur (species unnamed) in captivity were also refractory. The sensitivities of cells from Patas (Erythrocelus patas), Sykes (Cercopithecus mitis), Vervet (Cercopithecus contralis) and African Green monkeys (Cercopithecus aethiops) seemed higher than those of Rhesus (Macaca mulatta) and Cynomologus (Macaca cynomologus) monkeys but the small number of samples tested did not permit any conclusions to be drawn (Funahashi and Kitawaki, 1963). Differences in the susceptibility of red cells of individuals in the same species were reported by Cutchins (1962) and Funahashi and Kitawaki (1963).

Parameters: The interaction of measles haem-agglutinins with erythrocytes is rather slow. According to Norrby (1962a) the reaction continues for at least 30 minutes at 37°C and is influenced by pH values ranging from 4.8 to 10.1, the limiting factor being stability of the haemagglutinin. The pH stability range is broader than that of the infectious particle which is reported to be pH 5 to 9 (Black, 1959a; Musser and Underwood, 1960). The activity of the haemagglutinin at 4°C and room temperature was found to be four-fold and two-fold lower respectively than that at 37°C (Rosen, 1961). DeMeio and Gower (1961) obtained 8 to 16-fold lower titres at 4°C. Both groups used rhesus monkey (Macaca

mulatta) blood in their studies. Funahashi and Kitawaki (1963) did not find any difference in haem-agglutinin titres measured at 4°C and 37°C when they used African green monkey erythrocytes. However, when the reagents were prechilled and the test carried out at 4°C the titres were two to four-fold less. They, therefore, postulated that the interaction between the virus and erythrocyte was not mere adsorption.

The haemagglutination titre was linearly dependent on erythrocyte concentration. A final concentration of 2 to 3 x 10^5 erythrocytes per ml was optimal (Funahashi and Kitawaki, 1963). A concentration of 0.067 per cent erythrocytes in the reagent mixture was considered optimal by Norrby (1966).

Norrby (1962a) has carried out the most exhaustive studies of measles haemagglutination and he recorded many interesting observations. Haemagglutination was not observed in concentrations below 0.009 molar sodium chloride solutions. Divalent ions such as calcium and magnesium were more effective and the critical levels were 0.0012 molar calcium chloride and 0.0018 molar magnesium chloride. Norrby (1962a) pointed out that these data were similar to those recorded by Burnet and Edney (1952) for influenza virus and he contended that the electrolyte concentrations were necessary either for the virus-red cell interaction or for the general agglutinability of erythrocytes. He thought that the

similar requirements of electrolytes of measles and influenza haemagglutination systems which are otherwise dissimilar supported the latter hypothesis. On the other hand, the recent discovery of a salt-dependent haemagglutinin in measles cultures emphasizes the importance of ions in virus-erythrocyte interactions (Schluederberg and Nakamura, 1967).

Chemical aspects: Measles haemagglutination differs from that of the parainfluenza and influenza groups of viruses in several respects. The differences reflect certain basic dissimilarities in the nature of the haemagglutinins and also of the erythrocytes receptors with which they react.

Agglutination of simian erythrocytes by measles virus is not followed by elution. The salt-dependent haemagglutinin can be eluted from the agglutinated cells by resuspending the mixture in phosphate buffer solution, but no spontaneous elution occurs (Schluederberg and Nakamura, 1967). Attempts to elute the virus from erythrocytes by prolonged incubation at 37°C (Norrby, 1962a; Funahashi and Kitawaki, 1963), by exposure to higher temperatures (Norrby, 1962a), by alkalinization (Norrby, 1962a), by treatment with neuraminidase or by proteolytic enzymes (Funahashi and Kitawaki, 1963; Waterson, 1965) have been unsuccessful.

Measles haemagglutination is inactivated by treatment with trypsin and with urea and hence it was presumed

that the haemagglutinin was protein in character (Norrby, 1962b; Waterson et al., 1963).

The presence of lipid in the component is suggested by the effect of organic solvents such as ether, chloroform, acetone and methanol. Norrby (1962b) found that ether and chlorofrom extractions never gave more than a 50 per cent reduction of haemagglutination activity in the aqueous phase.

The haemagglutinin was precipitated at relatively low concentrations of acetone and methanol, further proof of its proteinaceous character. Precipitation maxima were found at 30 per cent acetone and 50 per cent methanol. With increasing concentrations of these solvents beyond the precipitation maxima, the amounts of haemagglutinins in the precipitate decreased but the activity reappeared in the supernatant fluids. These changes are different from those observed for influenza virus (Hoyle, 1962) and can be interpreted as splitting of the haemagglutinin lipoprotein.

The observation that sodium metaperiodate abolished measles haemagglutination and Newcastle disease virus haemagglutination suggested the presence of glycoprotein in the haemagglutinin molecule (Waterson et al., 1963).

Norrby (1962b) studied the effect of potassium periodate, hydrogen peroxide and iodine on the haemagglutinin and concluded that the action of these oxidizing agents was not on carbohydrate aldehydes.

Consequently, the haemagglutinin did not contain a polysaccharide moiety. He further opined that the effect of potassium periodate at the high concentrations used was probably on protein and not on carbohydrate. Iodine too probably had a similar effect. This surmise was substantiated by his studies on pH dependence. His results showed that the effect of iodine at low pH was mainly oxidation of the sulphydryl groups and at higher pH substitution. He also speculated on the possibility of iodine affecting the unsaturated bonds in the lipid part of the haemagglutinin molecule. The chemical nature of the haemagglutinin awaits elucidation and the existence of a heterogeneous group of particles with different grades of avidity adds to the complexity of the problem.

The erythrocyte receptors for measles virus are apparently not identical with those for other myxoviruses, and are probably not of a carbohydrate nature. This is supported by the evidence that neuraminidase has no effect on the absorption capacity of the erythrocytes (Funahashi and Kitawaki, 1963). Potassium periodate in a molar concentration of 0.0005 abolished the agglutinability of erythrocytes by myxoviruses (Hirst, 1948) but at this concentration it had only a slight denaturing effect on the erythrocyte receptors for measles agglutination (Norrby, 1962b). The action of trypsin in low concentrations points towards hydrolysis of

protein groups in the receptors (Norrby, 1962b). Formolized erythrocytes were not agglutinable by measles haemagglutinins (Peries and Chany, 1961). This original observation does not seem to have been confirmed or refuted.

Stability: Measles haemagglutinins exhibit considerable resistance to physical and chemical treatment. The results of studies by various workers on the thermal stability of the haemagglutinating activity are not strictly comparable because of the different preparations used (Norrby, 1962a; Peries and Chany, 1962; Waterson et al., 1963). Nevertheless it is well established that haemagglutination is a property which is heat stable relative to infectivity (Waterson, 1965). The haemagglutinating activity of the Tween 80-ether split particles is even more stable than that of the untreated material (Norrby, 1963; Enders-Ruckle, 1965).

There are few reports on the stability of the haem-agglutinins at different temperatures. Rosanoff (1961) found that the haemagglutinating activity of the crude virus preparation was lost in two hours at 56°C, in three to four days at 37°C, in seven days at -20°C and 5°C and in two weeks at -70°C. Funahashi and Kitawaki (1963) observed that haemagglutinin concentrated by centrifugation and suspended in phosphate buffer saline at pH 7.2 retained its activity at 37°C for nine days and at 4°C for at least three weeks.

They compared the thermostability of two preparations exposed to 56°C. The haemagglutinating activity of the "centrifuged concentrate" remained unaffected for the first six hours and then began to decline. The activity of the "carbowax concentrate", on the other hand, decreased quickly in the first two hours and then the inactivation slowed down. At the 40th hour the haemagglutinating activities were 32 and 256 times respectively lower than the original titres. Likewise, the titres of two other preparations, the carbowax concentrate and the Tween 80-ether antigen decreased to 32-fold and 8-fold respectively from the original values after 128 minutes irradiation with ultraviolet light.

Immunogenicity: Purified haemagglutinin is immunogenic (Norrby, 1964a; Gard, Carlstrom, Lagercrantz and Norrby, 1965). The haemagglutinin, separated from the nucleocapsid of the virus and the major part of the envelope lipids, stimulated the production of haemagglutination-inhibiting and neutralizing antibodies as effectively as the crude virus material. The two antibodies are probably identical (Kitawaki et al., 1964; Norrby, 1964a).

Inhibition: Antibodies to measles virus can be demonstrated by inhibition of haemagglutination (Peries and Chany, 1960; Cutchins, 1962; Black and Rosen, 1962; Togo, 1964). For this purpose the Tween-ether haemagglutinin is considerably more sensitive than the

untreated virus because the finer sub-division of the Tween-ether haemagglutinin requires less antibody per unit of haemagglutination activity (Funahashi and Kitawaki, 1963; Waterson et al., 1963; Waterson, 1965; Norrby, 1967).

HAEMAGGLUTINATION BY RINDERPEST VIRUS

To-date a haemagglutinin in rinderpest virus preparations has not been unequivocally demonstrated. Brotherston (1951$_b$) hinted at agglutination of sheep and fowl erythrocytes by lapinized rinderpest virus. Kuttler (1959), Huygelen (1960) and Plowright (1962a) using different virulent and attenuated strains of the virus at different temperatures, pH and virus concentrations failed to detect haemagglutinins against erythrocytes of a wide range of animal species. Leiss (1964), on the other hand, claimed to have observed clear cut haemagglutination of rabbit and guinea pig erythrocytes in titres up to 1:60 with the RBOK strain of rinderpest in bovine tissue cultures. He did not establish the specificity of the reaction by inhibition with rinderpest serum.

According to Plowright (1968) attempts by workers at the Farcha laboratory to demonstrate haemagglutinins in rinderpest cell cultures by physical methods and Tween-ether treatment were unsuccessful. It was, however, stated that incubation of Patas monkey

erythrocytes with rinderpest virus eliminated their reactivity to measles haemagglutinins. More recently, Provost, Queval and Borredon (1968) reported the presence of an intracellular haemagglutinin in rinderpest cultures which agglutinated Patas monkey erythrocytes. They described the parameters of the reaction and the characters of the particles. Their findings await confirmation.

HAEMAGGLUTINATION BY CANINE DISTEMPER VIRUS

Vladimirov (1949) claimed to have observed agglutination of human and frog erythrocytes by an egg-adapted strain of canine distemper virus. The reaction was allegedly inhibited by distemper convalescent serum. Uninfected chorio-allantoic membrane suspensions and chorio-allantoic fluids did not agglutinate. Haig (1949) observed, irregular partial agglutination of fowl and guinea pig erythrocytes with high concentrations of the Onderstepoort strain of egg-adapted distemper virus. Both reports have not been extended or confirmed. Gorham (1960) using another strain of the virus, failed to detect haemagglutinins against erythrocytes from nine species of mammals.

HAEMADSORPTION

Myxoviruses apparently mature on or near the surface of the host cell (Gey and Bang, 1951; Morgan, Rose and Moore, 1956; Niven, Armstrong, Balfour, Klemperer and Tyrrell, 1962). During maturation haemagglutinin is incorporated into the cell membrane and the modified membrane forms the outer coat of the progeny virus particle (Tyrrell, 1963). The phenomenon of haemadsorption initially discovered in monkey kidney cells infected with influenza A_2 virus (Vogel and Shelekov, 1957) is a useful marker of myxovirus cytopathogenesis in cell culture because it helps recognition of virus-infected cells much earlier than by any other known diagnostic procedure.

Haemadsorption of rhesus monkey erythrocytes by measles virus was first demonstrated by Mastyukova and Khait (1960). Hep-1 cells receiving a high input multiplicity of infection showed early cytopathic effects characterized by the appearance of syncytia within 24 hours. Foci of haemadsorption were demonstrated one to two days later using monkey erythrocyte suspensions. They tested a wide range of mammalian and avian erythrocytes and found that only simian cells were suitable. An 0.2 per cent suspension of erythrocytes and an incubation period of two hours at 37°C were recommended. The findings were confirmed and extended by Rosanoff (1961), Kohn (1962) and Kasahara, Makino, Sasaki and

Nakagawa (1963). The latter studied the parameters of the reaction and found them to be similar to those of the haemagglutination test except that positive results could be read in 30 minutes at 37°C.

Kohn (1962) noted an essential difference in haemadsorption by measles compared to haemadsorption by other parainfluenza viruses. Measles syncytia showed adherence of erythrocytes on the edge of the cell surface whereas in cell cultures infected with parainfluenza the entire cell surface was studded with red cells. Moreover, incubation of parainfluenza cultures for periods of three to five hours at 37°C caused lysis of the adsorbed cells (Kohn, 1962).

Very few published reports are available on measles haemadsorption and the basic mechanism of this irreversible linkage between the erythrocyte and the infected cells remains to be elucidated.

Haemadsorption in rinderpest and canine distemper cultures has not been demonstrated to date.

HAEMOLYSIS

Measles virus shares the property of haemolysis with other large myxoviruses such as Newcastle disease virus (Kilham, 1949; Burnet and Lind, 1950), mumps (Chu and Morgan, 1950) and parainfluenza type 1, 2 and 3 viruses (Sato, 1958; Sokol, Blaskovic and Krizanova, 1961). The haemolytic activity of these viruses has several features in common, the most important of which is that haemagglutination is a prerequisite for lysis despite the fact that the haemagglutinating activity of measles virus is chemically different from that of the other viruses (Norrby and Falksveden, 1964).

Measles haemolysis is apparently a function of the outer envelope of the virion because disintegration of the outer envelope resulted in diminution or complete loss of haemolytic activity (Norrby and Falksveden, 1964). Equilibrium centrifugation of the "small native haemagglutinin" showed that haemolytic activity was associated with a low density band, 1.25 grams per ml, which contained most of the lipids of the envelope (Schluederberg, 1962; Norrby et al., 1964a). A similar location of activity probably occurs in parainfluenza type 1, 2 and 3 viruses because extraction with lipid solvents entailed disappearance of haemolytic activity (Sokol et al., 1961; Rebel, Fontanges and Colobert, 1962; Kohn, 1965, 1967).

Measles haemolysis was first recognized by Peries

and Chany (1960) and its general properties were studied in detail by De Meio (1962), Norrby and Falksveden (1964) and Neurath and Norrby (1965a). Incubation of agglutinated cells at 37°C for periods over four hours caused lysis which was incomplete. Both agglutination and lysis were prevented by anti-measles serum, the latter even after agglutination had occurred (Norrby and Falksveden, 1964). Haemolysis was temperature-dependent and more sensitive to thermal inactivation than haemagglutination (Waterson, 1965). Haemolysis did not occur below 15°C but cells agglutinated. When the material was heated to 50°C haemolytic activity increased over a period of 30 minutes and then disappeared whereas haemagglutination was unaffected (De Meio, 1962). Haemolysis occurred within the range of pH 5.0 to 10.0 and optimally at pH 8.0 (Norrby and Falksveden, 1964; Waterson, 1965). Irradiation with ultra-violet light inactivated both activities at the same rate. Nitrous acid acted likewise in the first 15 minutes of treatment but thereafter haemolysin was affected at a faster rate (Neurath and Norrby, 1965a). Further differences in the chemical properties of haemolysin and haemagglutinin include ionic requirements, action of organic solvents, formaldehyde and photo-oxidation (Neurath and Norrby, 1965a).

The thermolability and thermal dependence of haemolytic activity suggested that the mechanism of

lysis was probably enzymatic and similar to that postulated for Newcastle disease and Sendai virus haemolysins (Neurath, 1964a, 1964b, 1964c, 1965; Norrby and Falksveden, 1964). Chemicals reacting with amino groups inactivated both haemolysin and haemagglutinin of Sendai virus, the former being the more susceptible. Abolition of haemolytic activity was also brought about by physical and chemical procedures influencing the aromatic amino acids. The inactivation was retarded by DIISO-propyl-fluorophosphate under certain conditions. Similar results were obtained with measles haemolysin (Neurath and Norrby, 1965a). In subsequent studies with concentrated and purified Sendai virus haemolysin direct evidence of enzymatic activities such as hydrolysis of beta-naphthyl-acetate, L-leucyl-beta-naphthylamine and DIISO-propyl-fluorophosphate was obtained and a hypothesis was mooted that the haemolytic activity was caused by lysozomal enzymes of host cell origin stimulated by the production of the virus particles (Neurath, 1964c, 1965; Neurath and Norrby, 1965b). A similar mechanism was thought to operate in measles haemolysis (Norrby, 1967).

Klemperer (1960) and Neurath (1965) studying Newcastle disease virus and Sendai virus respectively presented evidence to show that lysis of erythrocytes and tissue culture cells was due to disturbance of the electrolyte permeability of the cell membranes and

resulted in rapid swelling and disruption of the cells. Norrby (1964a) obtained similar results when he studied measles haemolysis. He found that there was an escape of potassium ions preceding release of haemoglobin and that phlorizin which has a stabilizing effect on cell membranes caused selective inhibition of haemoglobin release.

One notable difference between the Newcastle disease - mumps - parainfluenza group of the large myxoviruses and measles virus in respect of haemolytic activity is that pre-treatment of erythrocyte receptors with either Receptor Destroying Enzyme or influenza virus rendered them insusceptible to haemolysis by the large myxoviruses (Burnet and Lind, 1950) but did not prevent their lysis by measles haemolysin (Norrby and Falksveden, 1964).

Fusion-inducing factor(s): The large myxoviruses exhibiting haemolytic activity also caused lysis of cultured cells (Henle et al., 1954; Okada, 1958). In some instances, the activity seemed to be associated with syncytium-formation in vitro (Enders and Peebles, 1954; Brandt, 1961; Kohn, 1965, 1967) and in vivo (Enders et al., 1959). The particles associated with haemolysis and cell lysis were obtained from virions by disruption procedures and they exhibited identical physico-chemical characteristics indicating that they were expressions of the same effect (Norrby, 1967).

Measles virions rendered non-infectious by ultra-violet irradiation and non-infectious, low density particles obtained from measles cultures by physical methods induced syncytial formation in cell cultures (Toyoshima et al., 1960a; Schluederberg, 1962; Norrby et al., 1964a). The effect was non-transmissible. No viral antigens were demonstrated in the syncytia by immuno-cytological methods (Norrby et al., 1964a). The fusion-inducing effect was evident in five hours over a pH range of 8.3 to 8.8 and at incubation temperatures ranging from 30° to 37°C. (Cascarado and Karzon, 1965). The optimal values were 36°C and pH 8.3 (Norrby, Lagercrantz and Gard, 1966). The effect was prevented specifically by anti-measles serum (Cascarado and Karzon, 1965).

Both the haemolytic activity and the fusion-inducing property were inactivated within 20 minutes by heating to 56°C and were inhibited by phlorizin in 2×10^{-4} molar concentrations (Norrby et al., 1966).

The nuclear alterations that occurred in syncytia produced by the fusion factor were similar to those seen in syncytia associated with virus multiplication and were related to the mitotic cycle (Cascarado and Karzon, 1965). The detailed biochemical studies of Neurath (1964a, 1964b, 1964c, 1965) and Neurath and Norrby (1965b) on the mechanism of haemolysis by Sendai and measles viruses suggested that the underlying process

was enzymatic. Norrby (1967) justifiably assumed that syncytial formation was a protective device aimed at reducing injuries to the cell membrane by lytic enzymes located in the envelope of the virus. It is also possible that incorporation of cells into syncytial masses which was clearly observed by Thomison (1962) in his time-lapse microcinematographic studies with measles cultures is a mechanism that permits progressive infection in the presence of neutralizing antibody. Evidence stems from the work of Kisch, Johnson and Chanock (1962) and Coates and Chanock (1962) who showed that neutralizing antibody inactivated respiratory syncytial virus in the fluid phase only whereas syncytial formation and giant cell organization continued.

COMPLEMENT FIXATION

The introduction of the negative staining technique (Brenner and Horne, 1959), enabled the revelation of the ultrastructure of the myxoviruses and their division into two major groups on the basis of the diameter of the nucleocapsid (Waterson et al., 1961; Howatson, 1962) and led, in turn, to the morphological (Almeida and Howatson, 1963; Norrby, 1964a) and immunological (Waterson et al., 1963; Norrby, 1964b) assessment of the internal component of the measles virion and of rinderpest (Plowright et al., 1962) and distemper virions (Cruickshank et al., 1962).

MEASLES

Nature: Schluederberg (1962) and Schluederberg and Roizman (1962) attempted separation of measles virus particles in the caesium chloride density gradients and recognized two distinct classes of complement-fixing antigens on the basis of their buoyant densities. The bulk of the antigens was non-infectious, the infectious fraction accounting for only 3 per cent of the total complement-fixing activity. Their findings fitted in with the data demonstrating the presence of non-infectious, non-haemagglutinating, complement-fixing antigens in Newcastle disease virus (Schäfer and Rott, 1959) and Sendai virus (Hosaka et al., 1961).

Norrby (1964a, 1964b) characterized the two

fractions further. His Tween 80-ether or desoxycholate-split virus preparations yielded in caesium chloride density gradients two fractions both showing complement-fixing activity. One of the fractions was of low density, 1.29 to 1.30 grams per ml, and appeared to have been derived, at least in part, from the disintegrated envelope material. The low density fraction adsorbed on to erythrocytes. The other fraction was located in a sharp, heavier band, 1.32 grams per ml, and was entirely made up of nucleocapsids and did not adsorb on to erythrocytes.

More recently Numazaki and Karzon (1966) detected the existence of two infectious and one non-infectious complement-fixing antigens in measles-infected AV_3 cells. The antigens had different buoyant densities. One was a sharp band with a density ranging from 1.23 to 1.24 grams per ml and contained mature virions released from the cells. A second broad band contained heterogenous particles with densities ranging from 1.16 to 1.21 grams per ml and was thought to represent aberrant or precursor virions. The third was a sharp band having a density of 1.30 grams per ml, the so-called "H-CF" or heavy non-infectious antigen. It was serologically distinguishable from the other two antigens. The authors suggested that the "H-CF" antigen was analogous to the soluble (S) antigens of other myxoviruses because of its small particle size, early appearance in the

replication cycle, its non-infectious and non-haemagglutinating character and its high density which is a typical characteristic of nucleoprotein.

Another feature of the "H-CF" antigen that merits consideration is that antibody to it occurred only after virus replication and not after inoculation with inactivated virus (Numazaki and Karzon, 1966). Children immunized with inactivated measles vaccine developed HI antibodies but not S antibodies. On the other hand, S antibodies also appeared following exposure to natural measles (Numazaki and Karzon, 1966).

The antibodies to the two infectious forms of complement-fixing antigens were called V antibodies (Numazaki and Karzon, 1966), an unfortunate term because antisera to measles virus are also likely to contain haemagglutination-inhibiting and haemolysis-inhibiting antibodies which are also produced in response to the envelope antigens, the so-called V antigens.

The particle size of the non-infectious antigens was estimated to be 130 angstroms (Benyesh et al., 1958). Most studies have revealed that the particles associated with complement-fixing activity could be concentrated by high speed centrifugation (Toyoshima et al., 1960b; De Maeyer and Enders, 1961; Norrby, 1964b) but Numazaki and Karzon (1966) stated that their 'S' antigens could not be sedimented at 30,000 r.p.m. The discrepancy can be explained by the use of antigens of different grades

of purity.

Other cell disruption procedures, particularly sonication, are known to influence the distribution of particles in the density gradients (Schluederberg, 1968). It is possible that the different forms of antigens exhibiting complement-fixing activity occur along with disintegrated envelope material which accounts for the reported difference in the general properties of measles complement-fixing antigens. Machlowitz, Spicer, Buynak and Tytell (1961) found that while low concentrations of trypsin enhanced the activity probably by releasing intracellular antigen, high concentrations caused complete denaturation. Their findings were confirmed by Norrby (1967) but not by De Maeyer and Enders (1961) who reported that concentrations of up to 250 micrograms per ml had neither adverse nor salutary effects.

Likewise, the data on thermal stability of the antigens are not in agreement. De Maeyer and Enders (1961) and Norrby (1967) observed that incubation of virus preparations at 56°C for 30 minutes had no deleterious effect on complement-fixing activity but Girardi et al. (1958) reported reduction of activity. Formaldehyde (Girardi et al., 1958; DeWitt and Nook, 1960) and Tween 80 and ether treatment (Norrby, 1962b; Waterson et al., 1963) destroyed infectivity but preserved the complement-fixing activity.

Production: Enders and Peebles (1954) first detected

complement-fixing activity in primary simian and human cultures infected with measles virus. The titres were generally low and undiluted cell culture fluid had to be used for testing sera (Bech, 1959). In most culture systems, there seemed to be a linear relationship between the infectivity titres and complement-fixing activity titres. Schluederberg (1962) obtained titres of 1:16 or greater in Hep-2 cells in which the infectivity titres generally ranged from 10^7 to 10^8 $TCID_{50}$ per ml. Girardi et al. (1958), Norrby (1964b) and Mares, Drevo, Starek, Adam and Zavadova (1966) reported moderate to high titres in some cell systems. Kunita et al. (1963), on the other hand, experienced difficulty in obtaining even moderate levels of complement-fixing activity in a variety of human cell lines. They sonicated the infected cell sheets at the height of the cytopathic effect to release the intracellular antigens. Numazaki and Karzon (1966) demonstrated cell-associated complement-fixing activity in infected AV_3 cells within 16 hours; in other words, about 5 hours prior to the appearance of haemagglutinating and haemolytic activities.

RINDERPEST

Rinderpest virus was successfully propagated in cell culture twelve years ago (Plowright and Ferris, 1957) but to date there is no published account of the

characterization of its antigens in cell culture. Available data on the complement-fixation reaction in rinderpest are based on studies of the so-called soluble antigens in infected tissues from natural and experimental cases. The pertinent literature has been reviewed *in extenso* by Scott (1967) and somewhat less critically by Plowright (1968).

Complement-fixing activity in tissues of rinderpest-infected cattle was first demonstrated by Sasaki (1931). The antigen first appeared in the lymph nodes 24 hours after the onset of fever but not earlier. The findings were confirmed later (Nakamura and Wagatuma, 1937; Fukusho, Ishii and Takemoto, 1953; Taylor, 1959). The kinetics of the development of complement-fixing activity in the lymph nodes of experimentally-infected cattle were studied by Nakamura (1958). Antigen was demonstrable soon after the onset of fever and maximum titres were reached in 48 hours. The levels were maintained for another two to three days after which there was a decline which synchronized with the subsidence of the fever. The fall in titre progressed steadily until the eleventh or twelfth day when the antigen was no longer detectable. Similar data were furnished by Taylor (1959) and Stone and Moulton (1961).

Most studies have established a direct relationship between the intensity of the clinical reaction and the amount of antigen present in the lymph nodes of infected

cattle. Since the intensity of the clinical response is itself directly related to the growth of the virus, a positive correlation between the growth curve of the virus and the titres of the complement-fixing antigen in the lymph nodes was mooted, (Scott, 1967). The earlier findings of Fukusho et al. (1953) strengthened the hypothesis. They titrated the antigenic activity in the lymph nodes of experimentally-infected Holstein-Friesian and Japanese Black cattle which have different grades of innate resistance to rinderpest. The antigen titres were significantly higher in the more susceptible Japanese Black cattle which reacted more severely than the Holstein-Friesians. Complement-fixing activity was also readily demonstrable in the tissues of buffaloes, pigs and sheep infected with virulent strains of the virus (Nakamura, 1965). The activity appeared much earlier in cattle inoculated with the highly virulent Pendik strain of the virus than in cattle given the less virulent Kabete "O" strain (Boulanger, 1957b). A similar correlation between the virulence of the strain and the degree of antigenic activity in the lymph node tissue of infected cattle was noted by Provost and Borredon (1963).

Nakamura and Macleod (1959) examined the tissue of rinderpest-infected sheep for complement-fixing activity. There was no clear evidence of any relationship between the severity of lesions and the titres of antigen. They

concluded that infection in sheep was relatively milder than in cattle and that the propagation of virus was much lower. Barber and Heuschele (1964) were able to detect complement-fixing activity in only one of six American sheep experimentally infected.

Complement-fixing antigens of rinderpest virus were demonstrated in a number of experimental hosts such as sucking mice (Imagawa, 1965), rabbits (Nakamura and Goto, 1941; Reisinger, Mun and Lee, 1954), fowl embryos (Cooper, 1946; Nakamura and Kishi, 1952; Nakamura, Kishi and Kiuchi, 1955) and fowl embryo tissue cultures (Nakamura, Motahashi and Kishi, 1958).

Nakamura (1939, 1958) made quantitative studies of the distribution of complement-fixing antigens in the tissues of experimentally-infected cattle. Highest titres were obtained from lymphoid tissues and alimentary mucosa. Scott (1964, 1967) defined the optimal period for the collection of satisfactory samples from rinderpest-infected animals for demonstration of complement-fixing activity. The recommended period was the third to the sixth fever day (Scott, 1964). Samples taken after the onset of diarrhoea were not satisfactory (Scott and Brown, 1961). Likewise, samples from dead carcases were of doubtful value because a significant number of deaths occurred after the disappearance of the antigens (Scott, 1964).

Details of preparing complement-fixing antigens of

good titres from rinderpest-infected tissues have been furnished by several workers (Boulanger, 1957b; Nakamura, 1958; Cowan, 1961; Moulton and Stone, 1961) and the relative merits of the techniques used have been discussed by Macleod and Scott (1963) and Scott (1964, 1967).

Data on chemical characterisation of the antigen are meagre. The crude tissue antigen was thermostable (Nakamura, 1958) and was not denatured by organic lipid solvents (Boulanger, 1957b).

CANINE DISTEMPER

The complement-fixation test for the detection of antigens has been widely applied in the diagnosis of dog distemper but little is known of the nature of the antigens that are associated with the reaction. Until the advent of tissue culture techniques for the propagation of distemper virus, the sources of antigen for the complement-fixation test were crude suspensions of tissues from infected dogs or ferrets.

Laidlaw and Dunkin (1931) first demonstrated complement-fixing activity in the spleens of experimentally infected ferrets and they established a tentative correlation between infectivity and complement-fixation titres. They commented on the anti-complementary activity of some of their preparations and drew attention to the problem of non-specific fixation by some animal

sera. Their findings were neglected for two decades; instead research on distemper was geared to technological improvements in the demonstration of inclusion bodies in clinical material and in assessing their usefulness and reliability in distemper diagnosis.

Drager and Schindler (1951) detected complement-fixing activity in a variety of tissues and body fluids collected from a dog 22 days after infection. Bindrich (1954) conducted an extensive study using 3,519 organs from 1,292 infected dogs and appraised the distribution of antigenic activity in different tissues. Liver, lung, spleen, kidney, lymph nodes, tonsillary tissue and mucosa of the urinary bladder contained demonstrable antigens but the highest titres were obtained in mesenteric lymph nodes and spleen. The recommended sampling period was between the fourth and twelfth day after exposure: a similar pattern of distribution of the antigens was noted in ferrets and good yields were obtained between the fifth and tenth day after experimental infection (Bindrich, 1954).

Mansi (1955) is reported to have used the complement-fixation test extensively to determine the serological specificity of distemper virus (Gorham, 1960). His method of preparation of the antigen from canine and ferret tissues was similar to that described by Cassals and Palacios (1941) for arboviruses. Good titres were obtained from spleens collected ten to

twelve days after infection.

After the adaptation of distemper virus to the developing fowl embryo (Haig, 1948; Cabasso and Cox, 1949) attention was focussed on fowl embryo tissues as an antigen source. Mansi (1955) failed in his attempts to extract the antigen from infected chorioallantoic membranes but he was able to liberate it by sonication. He did not detect antigenic activity in the allantoic fluid or in the whole embryo.

Morris, Avlisio and McCoun (1955) demonstrated the antigen in the allantois fluid and in the chorio-allantoic membranes of embryos 15 days old which were inoculated six days previously. Anticomplementary activity of the preparations was obviated by freezing and thawing and sonication followed by clarification in the ultracentrifuge. The antigen so obtained titred 1:64. The findings were confirmed and amplified by Fastier (1956) and Prier, Wright and Kalter (1962). An improved procedure involved absorption of the membrane suspensions with basic ion exchange resins (Fastier, 1956).

Karzon, Gillespie and Bussell (1961) and Bussell and Karzon (1962, 1965a) discussed the yields of complement-fixing antigens from ferret, canine and fowl embryo cell cultures. Notwithstanding low infectivity titres of between 1.7 and 3.2 $\log_{10}$ TCD_{50} per ml, the ferret cultures showed higher antigenic activity than the fowl embryo fibroblast cultures. The difference was

attributed to the thermolability of the latter antigen (Bussell and Karzon, 1965_b).

Data on the thermostability of the antigenic preparations are not always in agreement. Laidlaw and Dunkin (1931) found that the complement-fixing activity in crude suspensions of infected spleen was reduced by heating to 50°C for one hour and was totally abolished at 60°C in 30 minutes. On the other hand, Bussell and Karzon (1965_a) reported that the antigen derived from mammalian cultures was stable at 56°C. Bullier (1929) had claimed earlier that the antigenic activity in the brain tissue of naturally infected dogs withstood boiling for one hour (Laidlaw and Dunkin, 1931). It is possible that complement-fixing activity is associated with particles of different physico-chemical properties. Chemical characterization of the soluble antigens of distemper virus is awaited.

PRECIPITATING ANTIGENS

In the "medipest" group of viruses the demonstration of precipitating antigens in infected tissues has been confined to rinderpest and distemper.

RINDERPEST

The existence of rinderpest precipitinogens in extracts of infected tissues was first revealed by Ruppert (1919) and confirmed by Daubney (1928) who attempted to precipitate the antigens in the infected tissues with alcohol. He demonstrated a flocculation reaction between his antigen preparation and the sera from convalescent and recently vaccinated animals. Later workers were unable to repeat the experiments (Scott, 1964).

Using the Ouchterlony agar-gel diffusion technique, White (1958a, 1958b) unequivocally demonstrated a single line of precipitation between rabbit hyperimmune anti-rinderpest serum and infected bovine lymph node tissue. He failed to obtain a reaction with lymph nodes from convalescent cattle and rabbit sera.

Stone (1960) reported two forms of soluble antigens in infected cattle lymph node extracts; one that migrated fast was inactivated by heating to 56°C for 30 minutes. The other was heat stable. White and Cowan (1962) separated a soluble antigen from infectious particles by high speed centrifugation and found that it

was thermolabile. Scott and Brown (1961) mentioned the presence of two lines of precipitation "on occasion" but usually the reaction between infected cattle lymph node extracts and hyperimmune rabbit serum resulted in one band of precipitation.

Recently Japanese workers (Ishii, Tokuda and Watanabe, 1964) detected three different antigens in lyophilized lymph node material extracted with veronal buffer. Two withstood boiling for 30 minutes while the third, a slowly migrating antigen, was thermolabile. The antigens were not sedimented by centrifugation for 60 minutes at 40,000 r.p.m. It merits mention that they used bovine hyperimmune serum in their tests. They further noted that rabbit lymph node extracts contained only a single fast-moving heat-stable antigen (Ishii et al., 1964). The disparate findings regarding the number of antigens, their electrophoretic mobility and thermal stability might reflect differences in the strains of virus, the host system, sampling periods, the extraction procedures, the types of immune serum and the conditions of the test.

Brown and Scott (1960a) studied the kinetics of in vivo development of the precipitinogens of rinderpest virus in cattle. Sequential samples were obtained from prescapular lymph nodes by aspiration biopsy. The diffusible antigens first appeared 24 hours after onset of fever and persisted until the eighth day. Most

positive samples were taken between the third and fifth day of fever. Titration of antigenic activity at different intervals of infection revealed high levels on the third to the sixth day of fever. The antigens were not readily detectable after the eighth day of fever. There were, however, some differences in the precipitinogen titres of samples taken during the febrile period and shortly thereafter between animals that died of the infection and those that were slaughtered. In both groups, peak levels were attained on the fourth day of fever. The titres were maintained for a further two days in the slaughtered group and three to four days in the group that died. Animals killed ten days or later after the onset of fever were always negative whereas animals that died up to the twelvth day after the onset of fever remained positive. Precipitinogens were never detected after the thirteenth day. Scott (1967) therefore concluded that at a time when the majority of cattle die from rinderpest, appreciable amounts of diffusible antigens can be expected to be present in their lymph nodes. The 10 to 15 per cent of cattle that die late will not have antigens in their lymph nodes. Destruction of the precipitinogens in dead cattle due to autolytic changes in the carcase was slower than the inactivation of the virus (Scott, 1967).

Scott and Brown (1961) listed 18 other tissues in which the antigens were demonstrable but none was so rich

in antigens as the lymph nodes. It is interesting to note that the lung tissue and the brain contained no antigens, because these are frequently positive in distemper (Fraser, 1966). A point of additional interest is that the development and distribution of precipitinogens and complement-fixing antigens in cattle are similar (Scott, 1967). No comparable data are available for rinderpest in goats and rabbits.

Scott (1967) warned against overemphasis of the reliability of the agar-gel diffusion test in the detection of rinderpest antigens in outbreaks in enzootic regions. There was a direct relationship between the presence of precipitinogens in the tissues and the nature of the clinical reaction of the animal. In cattle infected with three strains of descending virulence the percentage of positive samples was 50, 32 and 6 (Scott and Brown, 1961). The frequent occurrence of strains of rinderpest virus that are naturally attenuated for cattle in enzootic countries is well documented (Scott, 1964). Examples include the strains isolated and studied by Provost and Borredon (1963) which did not stimulate the production of precipitinogen in the tissues of affected cattle.

CANINE DISTEMPER

The precipitation test as an aid in the diagnosis of distemper was first applied by Mansi (1957). In

Ouchterlony agar-gel diffusion plates he noted a single line of precipitation between crude aqueous extracts of infected lymph node tissue and hyperimmune canine distemper serum. Later, he modified the test by using microscope slides coated with agar (Mansi, 1958). Besides the economy in the use of antigen and immune serum, the speed and efficiency of the reaction improved considerably in the slide test. Distinct precipitation lines were detected within one to three hours as opposed to the 5 to 18 hours in the plate test. Mansi (1958) studied the distribution of the antigen in the different tissues of infected animals and recommended lymph node, spleen and lungs for diagnostic sampling because their content of the antigen was high.

Fraser (1966) used the test successfully in the diagnosis of dog and mink distemper. The distribution of the precipitinogens in the different tissues of the infected dog and mink was similar, the highest percentage of positive reactions being obtained from lungs and mesenteric lymph nodes. The antigen was demonstrated readily in the spleen tissue of the dog but not of the mink. He used hyperimmune anti-rinderpest serum derived from cattle, goats and rabbits and obtained a single line of precipitation in Ouchterlony plates.

White and Cowan (1962) separated the precipitinogens from the infectious virus particles by ultracentrifugation. They found that the pre-

cipitinogens of distemper and rinderpest were similar in regard to properties such as thermolability, absorption on DEAE-cellulose and precipitability by ammonium sulphate (White and Cowan, 1962).

ANTIBODY

Antibodies to the "medipest" viruses are demonstrable by neutralization tests, complement-fixation tests and haemagglutination-inhibition tests (Stokes, Reilly, Hillman and Buynak, 1961; Cutchins, 1962; Black and Rosen, 1962; Norrby et al., 1963b). Ancillary tests such as the haemadsorption-inhibition (Mastyukova and Khait, 1960; Rosanoff, 1961), mixed agglutination (Fagraeus and Espmark, 1961; Barrow, Milgrom, Karzon and Witebsky, 1963), and haemolysis-inhibition tests (Saburi and Matumoto, 1966) are also available for the recognition of antibodies to measles. Although their specificity is unquestioned (Norrby, 1967) the relative sensitivities of the ancillary tests in the detection and quantitation of antibodies in comparison to the established tests have not been fully determined.

HAEMAGGLUTINATION - INHIBITION TEST

Measles: The specificity and simplicity of the haemagglutination-inhibition test in the serological confirmation of a diagnosis of human and simian measles was established independently by De Meio and Gower (1961), Rosanoff (1961) and Rosen (1961) soon after the discovery of measles haemagglutination (Mastyukova and Khait, 1960; Peries and Chany, 1960). The variables of the test have been worked out in detail (Rosen, 1961; Funahashi and Kitawaki, 1963; Waterson et al., 1963) and

a workable procedure which was easy to carry out and which gave consistently satisfactory results was established (Rosen, 1961; Fulginti and Kempe, 1963).

The source of the haemagglutinin was of paramount importance because it alone was the largest single factor controlling the sensitivity of the test. The haemagglutinin preparation of choice was the split product obtained by Tween 80-ether treatment of the native haemagglutinin from measles cultures (Norrby, 1962b; Waterson et al., 1963). The treatment led to an enhancement in haemagglutination activity and also caused a two to eight-fold increase in the sensitivity of the inhibition test (Funahashi and Kitawaki, 1963; Enders-Ruckle, 1965; Norrby, 1967). The stability of the split product was also superior to that of the native haemagglutinin (Togo, 1964; Norrby, 1967).

The salt-dependent haemagglutinin described by Schluederberg and Nakamura (1967) appears to be as sensitive as the Tween 80-ether product and preliminary comparative studies (Schluederberg and Nakamura, 1967) indicated that better antibody titres were obtained with the salt-dependent haemagglutinin. The value of the salt-dependent haemagglutinin reaction awaits confirmation; a reference to its application is conspicuously absent in Norrby's (1967) otherwise excellent and comprehensive review of measles.

The technique of the measles haemagglutination-

inhibition test originally outlined by Rosen (1961) is followed in most laboratories with minor modifications relating to the volumes of the reagents. A micro-technique devised by Takatsy (1950) or its modification by Sever (1962) has been used in some studies (Waterson et al., 1963). Norrby (1967) recommended an 0.17 per cent concentration of erythrocytes as optimal but most workers have preferred 0.5 to 1.0 per cent suspensions to facilitate quicker sedimentation and earlier reading of results (Funahashi and Kitawaki, 1963; Kumita et al., 1963; Waterson et al., 1963).

Meyer et al. (1962) who carried out a systematic study of serological standards in measles stated that the maximal reaction between the antibody and haem-agglutinin was reached after incubation for one hour at 4°C or 25°C. Norrby (1964c) found that refrigeration overnight after prior incubation of the serum-haemagglutinin mixtures for one hour at room temperature often gave a two to four-fold increase in serum titres.

Pretreatment of sera for removal of complement, heterologous agglutinins and non-specific inhibitors was a prerequisite of the test but the procedures did not outweigh the test's simplicity. The procedure recommended for the removal of the inhibitors was to treat the inactivated sera with a 25 per cent suspension of acid-washed kaolin in phosphate buffer solution at pH 7.2 (Rosen, 1961; Gutchins, 1962; Togo, 1964) and

to centrifuge the mixture at 3,000 r.p.m. for 15 to 20 minutes after incubation at room temperature for 20 minutes. The supernatant fluid was then removed for the test. Kunita et al. (1963) used treatment with cold acetone as an alternative method, with success, but the procedure was not feasible in large-scale serological studies. No data are available regarding the nature and degree of non-specific inhibition in human and animal sera for measles agglutination.

Waterson et al. (1963) treated their sera with M/90 potassium periodate solution to inactivate nonspecific agglutinatinins but a simpler and more efficient procedure was to adsorb, preferably at 4°C, inactivated sera with a packed or 50 per cent suspension of monkey erythrocytes and then to centrifuge the samples (Rosen, 1961; Runahashi and Kitawaki, 1963; Norrby, 1967). For human sera one hour incubation at 37°C with equal volume of 5 per cent monkey erythrocytes was considered sufficient by Kunita et al. (1963).

The sensitivity of the measles haemagglutination test is well established. Even with crude virus preparations the sensitivity was found to be on a par with that of the neutralization test both in terms of antibody titres and in the percentage of positive samples detected (Cutchins, 1962; Black and Rosen, 1962; Togo, 1964). When purified haemagglutinins were used the titres were usually five to ten times higher than the

neutralization titres (Norrby, 1963b; Waterson et al., 1963; Enders-Ruckle, 1964).

Parallel studies have revealed good correlation between titres obtained in the neutralization and haem-agglutination-inhibition tests (Black and Rosen, 1962; Cutchins, 1962; Krugman et al., 1965; Millian et al., 1965). Consequently speculation arose as to whether or not the two antibodies were identical (Norrby, 1967). In support of this thesis Norrby (1967) cited his own experimental data (Norrby, 1964a; Norrby et al., 1965a) and those of Kitawaki et al. (1964) which showed that purified haemagglutinins stimulated the production of antibodies with both reactivities. In one study on children immunized with an inactivated measles vaccine a correlation coefficient of 0.86 was recorded between the titres of antibodies in the two tests (Norrby et al., 1963b). Further, similarities in the induction, maintenance and fall of the neutralization and haem-agglutination-inhibiting antibodies in populations following natural or vaccine-induced measles have been reported (Black and Rosen, 1962; Kunita et al., 1963; Togo, 1964; Enders-Ruckle, 1964; Krugman et al., 1965).

The measles haemagglutination-inhibition test is the serological procedure of choice in study of measles serological diagnosis and immunity. It is the simplest and most practical test to carry out and requires minimum reagents (Krugman et al., 1965). The use of a standard

serum is not necessary in this test. Further, the test is as sensitive an indicator of measles antibody as the neutralization test (Norrby, 1967).

Rinderpest: A direct haemagglutination-inhibition test using rinderpest antigens and the homologous serum has not been developed. Provost and Borredon (1968) described an intracellular haemagglutinin in rinderpest-infected cell cultures and characterized some of its physico-chemical properties. The haemagglutinin was active against Patas monkey (Erythrocebus patas) erythrocytes but the small yield of the haemagglutinin obtained from infected cells did not permit confirmation of its specificity by an inhibition test.

The discovery of the existence of two main antigens in the measles virion, namely, the haemagglutinin and the complement-fixing antigen, permitted further analysis of the postulated serological relationship between measles and rinderpest. Using Tween-ether haemagglutinin, Waterson et al. (1963) observed specific inhibition of measles haemagglutination by rinderpest antisera. Their findings were confirmed and extended by Enders-Ruckle (1964), Bogel, Enders-Ruckle and Provost (1964) and Norrby (1967). In all rinderpest sera examined antibodies reactive with measles haemagglutinins were detected; higher titres were found in cattle infected with virulent virus than those receiving attenuated virus although the rinderpest neutralizing

antibody titres were of the same magnitude in both (Norrby, 1967).

Bogel, Enders-Ruckle and Provost (1964) carried out detailed studies on the application of the measles haem-agglutination-inhibition test in the detection of rinderpest antibodies in the sera of West African cattle. The cattle sera contained no non-specific inhibitors. Significant titres of antibodies reacting with measles Tween 80-ether haemagglutinins appeared in cattle 9 to 12 days after inoculation with virulent or attenuated rinderpest virus and the titres persisted for weeks or months. By means of a modified test they demonstrated rinderpest virus in infected lymph nodes of cattle.

In another study Bogel, Provost and Enders-Ruckle (1966) examined 1,967 sera from cattle reared in rinderpest-free areas and found haemagglutination-inhibiting antibodies in the serum of only one calf. On the other hand, they sometimes found no haem-agglutination-inhibiting antibodies in the sera of cattle vaccinated with attenuated strains of rinderpest. Neutralization antibodies were, however, detected and they appeared earlier than the haemagglutination-inhibiting antibodies (Bogel et al., 1966).

In a similar study carried out in Northern Nigeria, Rowe, Zwart and Kouwenhoven (1967) measured the levels of antibodies to rinderpest in 118 randomly selected cattle using the measles haemagglutination-inhibition and

rinderpest neutralization tests. The cattle had been previously vaccinated against rinderpest on several different occasions. Both tests gave positive titres in 93 cases and negative titres in 7. An interesting observation that emerged was that 9 sera having demonstrable measles haemagglutination-inhibiting antibodies had no rinderpest neutralizing antibodies. The authors took care to remove possible non-specific inhibitors in the cattle sera by treatment with kaolin.

Canine distemper: As with rinderpest, an haemagglutination-inhibition test using measles Tween-ether antigen has been developed (Waterson et al., 1963; Enders-Ruckle, 1964) but its value in serological diagnosis of distemper seems to be limited (Norrby, 1967). Antibodies reactive with measles haemagglutinins were demonstrated in a few samples of sera from dogs immunized with Rockborn's attenuated virus but not with the virulent Snyder Hill strain (Norrby, 1967). The problem merits detailed investigation using different wild and attenuated strains of distemper virus.

HAEMADSORPTION-INHIBITION TESTS

Myxoviruses having the property of haemagglutination may be detected in cell cultures by adsorption of specific erythrocytes on to the surface of infected cells. This phenomenon of haemadsorption was initially recognized in measles cultures independently by

Mastyukova and Khait (1960) and Rosanoff (1961). Adsorption was specifically inhibited by pretreatment of infected monolayers with anti-measles serum (Kohn, 1962; Kasahara et al., 1963).

As a diagnostic tool haemadsorption helped early recognition of virus growth. Its usefulness was matched only by the fluorescent-antibody technique but it was a far simpler test although not so specific per se. Specificity was provided by haemadsorption-inhibition.

No quantitative studies seem to have been carried out on the measles system and it is difficult to relate the degree of inhibition of haemadsorption to the concentration of antibodies in a sample of anti-measles serum. The technique of quantitative haemadsorption described by Finter (1964) in which the adsorbed erythrocytes were lysed with distilled water and the amount of haemoglobin released was taken as index of antigenic activity on the surface of vaccinia-infected cells may be useful in assessing the degree of haemadsorption-inhibition. Finter (1964) used the technique for the assay of interferon.

MIXED AGGLUTINATION TESTS

The mixed agglutination test was first successfully applied to cell cultures to detect blood group antigens (Hogman, 1959). The cultured cells were sensitized

with antibody to the surface antigens. The antigen-antibody complex was then visualized by adsorption with erythrocytes coated with anti-gamma-globulin reactive with the antibody used to sensitize the antigens on the cells. Barron et al. (1963) demonstrated measles antibody by this method. Tanned human "O" group erythrocytes were incubated with a solution of human gamma-globulin at room temperature for 30 minutes. The coated erythrocytes were washed thrice and finally resuspended as a 0.5 per cent suspension which was then conjugated with an equal volume of a potent rabbit anti-human gamma-globulin serum at room temperature for 30 minutes. The complex was washed thrice and used as 0.25 per cent suspension in the test. Infected monolayer cultures were treated with serial two-fold dilutions of anti-measles serum and incubated for one hour at room temperature. The serum was then removed and the monolayers were washed thrice with phosphate buffer saline. The indicator system was added and the cultures were incubated for one hour at room temperature. Haemadsorption was taken as evidence of the presence of antibody.

Barron et al. (1963) carried out a comparative study of the efficiency of mixed agglutination, complement fixation and haemagglutination-inhibition tests and obtained more positive results and higher titres with the mixed agglutination test. Norrby (1967)

questioned the specificity of the test without giving any valid reasons. A possible artifact is the non-specific union between antibodies in non-immune sera and uninfected cell antigens. A system of controls in which infected and uninfected cells are treated with pre-inoculation and postinoculation sera seems essential.

Fagraeus and Espmark (1961) applied the test with success in the quantitation of distemper antibodies but their findings have not been confirmed.

The test offers little or no advantage over the much simpler haemagglutination-inhibition test in measles serodiagnosis.

HAEMOLYSIS-INHIBITION TESTS

Concentrated measles virus suspensions and crude haemagglutinins caused partial lysis of monkey erythrocytes on prolonged incubation at 37°C and the effect was prevented by anti-measles serum (Norrby and Falksveden, 1964). A haemolysis-inhibition test therefore was developed by Sabusi and Matumoto (1966) for the assay of measles antibody. They obtained good correlation between positive results and antibody values in comparative studies on post-vaccinal human sera with haemolysis-inhibition, complement-fixation and neutralization tests. Non-specific haemolysis-inhibition was evident with some non-immune sera. In addition the test is inconvenient for routine use

because of the long incubation period required and the necessity for sphectrophotometric analysis of samples for detection of cyanmeth-haemoglobin.

COMPLEMENT-FIXATION TESTS

Measles: The measles complement-fixation test is a typical virus complement fixation procedure. Two methods are available, the so-called "tube" and "drop" methods. Both give satisfactory and reproducible results (Enders, 1964). The "tube" method is often recommended for the accurate comparison of quantitative data from different laboratories whereas the economical "drop" technique is widely used in titration studies of large numbers of samples, (Svedmyr, Enders and Holloway, 1952).

The variables of the test have not been studied in detail (Enders, 1964). Most workers preferred two units of antigen (Beck, 1959; Meyer et al., 1962) but Kunita et al. (1963) obtained reproducible results and often better titres with four units. Meyer et al. (1962) investigated the problem systematically and concluded that the differences in antibody titre resulting from the use of two to eight units were trivial.

Anticomplementary activity of sera was more common than that of crude antigen preparations; the anti-complementary activity of the antigens tended to disappear after heating to 56°C for 30 minutes (Ruckle and

Rogers, 1957). A simple method for removing anti-complementary activity of stored sera was to subject them to two cycles of heat-inactivation in the presence of bovine serum albumin (Enders, 1964).

Non-specific fixation of the antigen by non-immune sera was another artifact but the nature of the phenomenon is not known (Warren et al., 1960; Rao, 1963).

The standard technique is a modification of Kolmer's original method (Kolmer, Sprawling and Robinson, 1952) in which two full units of guinea pig complement are employed, with a fixation period of 16 to 18 hours overnight in the refrigerator. The reagents are dispensed in 0.1 ml amounts. Even antibody titres as low as 1:2 are regarded as evidence of past contact with measles virus (Enders, 1964).

The measles complement fixation test is generally regarded as less sensitive than neutralization and haemagglutination-inhibition tests (Norrby et al., 1963b; Ruckle-Enders, 1965). The complement-fixing antibodies appeared somewhat later and persisted for shorter periods than the neutralizing antibodies in the sera of patients convalescent from (Cutchins, 1962; Black and Rosen, 1962) or vaccinated against measles (Mcrumb et al., 1961a; Krugman et al., 1965). In early convalescence the antibody levels were comparable (Ruckle and Rogers, 1957; Stokes et al., 1961a, 1962)

but later the complement-fixing antibody titres were reduced (Stokes et al., 1961a; 1962). The discrepancy arises either from a change in the reactivities of the two antibodies with time after immunization or to the existence of two different classes of antibodies reactive with the two forms of complement-fixing antigen (Norrby, 1967). Henle et al. (1948), Watson (1952), Traver, Northrop and Walker (1960) observed that in mumps infections of man and experimental animals the complement-fixing antibody to the so-called S or nucleocapsid antigens appeared earlier than the complement-fixing antibody directed against the V antigens or the haem-agglutinins. Possibly measles complement-fixing antibodies behave likewise because Norrby (1967) discovered that in the early phase of recovery from natural measles the complement-fixing and neutralizing antibody titres ran parallel whereas following immunization with inactivated vaccines the neutralizing titres were usually several times higher. The recent finding (Numazaki and Karzon, 1966) that the antibodies to the highly purified nucleocapsid antigens from measles cultures were not detected after immunisation of school children with inactivated vaccines but were present following subsequent exposure to natural measles strengthens the hypothesis.

The fact that complement-fixing antibodies appear during the early convalescence period is of distinct value in measles diagnosis. Examination of paired sera

collected from early and late convalescence phase of the infection will show a significant rise in antibody titres.

Rinderpest: Complement-fixing antibodies in the sera of cattle convalescent from rinderpest were first recognized by Petroff in 1922 (Curasson, 1932) and were unequivocally demonstrated by Nakamura (1936, 1958), Cooper (1946), Walker et al. (1946), Nakamura et al. (1955) and Moulton and Stone (1961).

According to Nakamura (1958), the early Japanese investigators used extracts of desiccated infected bovine lymph node tissue as antigen. The fixation period was four hours at 4 to 7°C and the complement dilution variation was favoured. Demonstration of antibodies depended upon the presence of heat-labile factors in the serum (Nakamura, 1958; Nakamura and Macleod, 1959).

Walker et al. (1946) tried a serum dilution method without much success. They inactivated the sera. Cooper (1946) who also used heated sera did not titrate the antibodies. Instead, he titrated the virus which was obtained from embryonic fluids of fowl embryos inoculated chorio-allantoically. Antibodies were demonstrable in sera of cattle within 9 to 17 days of inoculation and persisted for up to six months (Cooper, 1946). Brotherston (1951_b), on the other hand, had no success with inactivated ox sera and infected goat and rabbit lymph node extracts.

Pellegrini and Guarini (1952) succeeded in demonstrating antibodies in convalescent cattle after diluting the sera with a 1.5 per cent solution of sodium chloride to remove non-specific reactivity. The antigen consisted of centrifuged mixtures of infected bovine lymph node tissue and gastric mucosa and it was heated at 56°C for 30 minutes before use. They obtained titres of 1:30 to 1:40 in immune animals.

Nakamura and Ishii (1953a, 1953b) and Nakamura and Macleod (1959) employed the complement dilution technique. The sera which were not inactivated were tested against infected and uninfected lymph node tissue, the optimal dilution of the positive antigen having been determined by prior titration against a known positive serum. Fixation proceeded at 4°C for 4 hours. An arbitrary formula that took into consideration the degree of non-specific haemolysis in the normal antigen scales was used to determine the specific antibody values, a questionable procedure.

Moulton and Stone (1961) also used the complement-dilution technique but inactivated the sera. Fixation occurred at 4°C after 18 hours. Addition of small amounts of unheated normal ox serum did not influence the antibody titres.

The conclusion that stems from the foregoing account is that the rinderpest complement-fixation test for the detection of antibodies is laborious, time-

consuming and difficult to perform and the results are also sometimes difficult to interpret. It is, therefore, not recommended as a routine test for the serological diagnosis of rinderpest (Scott, 1967).

Complement-fixing antibodies first appeared in the sera of cattle 9 to 17 days (Cooper, 1946), 7 to 14 days (Nakamura, 1958) and 10 days (Moulton and Stone, 1961) after experimental inoculation of cattle. Cooper (1946) noted peak titres between 14 to 18 days and the antibodies persisted for up to 6 months. Nakamura (1958), in contrast, reported that they disappeared within a few days of recovery. He did not find any correlation between the titre of complement-fixing antibodies and the degree of clinical reaction in cattle; some animals did not develop any detectable titres in spite of their manifesting clinical illness (Nakamura, 1958). On the other hand, Scott (1967), believed that a direct relationship existed between the severity of the clinical reaction and the ability to produce complement-fixing antibodies. According to his data (Scott, 1967) the development of complement-fixing activity in the serum closely followed that of the neutralization activity, the difference in the time of onset of the two activities being one day only. Virus and both types of antibody could be isolated from the same blood sample 7 to 9 days after the onset of fever (Scott, 1967).

In rinderpest-inoculated rabbits complement-fixing

antibodies were readily demonstrable on the 7th day or after. Peak titres were reached between 15 to 20 days and antibodies persisted for up to 70 days (Nakamura, 1958).

Canine distemper: There is paucity of information on the variables in the distemper complement-fixation test and also on its value in serological diagnosis. Much of the early work was aimed at the demonstration of complement-fixing activity in the tissues of infected dogs and ferrets with the objective of titrating virus activity (Dedie, 1951; Bindrich, 1954; Mansi, 1955; Dedie et al., 1957).

Many workers experienced difficulty with the anti-complementary properties of dog serum (Gorham, 1960). Procedures such as repeated freezing and thawing, centrifugation, or the addition of normal canine serum or guinea pig complement before heat-inactivation were recommended by Mansi (1955). He also suggested that sera be separated from the clot within an hour after bleeding and immediately frozen or lyophilized. These procedures are not feasible in quantitative studies involving large numbers of samples.

Sera were usually inactivated at 56°C for 15 to 30 minutes (Laidlaw and Dunkin, 1931; Pyle and Brown, 1935; Dedie, 1951; Dedie and Klapotke, 1951, 1952; Drager and Schindler, 1951; Bindrich, 1954; Cammand, Mackowiak, Joubert and Goret, 1956; Fastier, 1956;

Kilham, 1956). Higher temperatures for shorter intervals such as 60°C for 5 minutes (Savan and Brandly, 1950) or 64°C for 15 minutes (Mansi, 1955) were favoured when the anticomplementary activity of the sera was marked. Mansi (1955) preferred the use of unheated sera if they were not anticomplementary.

The time and temperature of incubation of serum-antigen mixtures varied widely. Morris et al. (1955), Prier et al. (1956), Cabasso et al. (1957) and others found overnight fixation at 4 to 6°C to be suitable. On the other hand, early workers who used infected tissue suspensions as the source of antigen were successful in demonstrating antigenic activity after primary incubation at 37°C for 30 minutes (Laidlaw and Dunkin, 1931; Savan and Brandly, 1950; Dedie, 1951; Mansi, 1955). Few attempted titration of the antibodies.

Dedie (1951) and Mansi (1955) emphasized the need for using different dilutions of complement in the test. Corvazier and Lasfarques (1952) were apparently successful in detecting both virus and antibody in the conglutination-complement absorption test which they recommended as being superior to the complement-fixation test.

NEUTRALIZATION TEST

Measles: The ease with which adapted strains of

measles virus can be propagated in cell lines simplified the procedures for *in vitro* serum-virus neutralization tests and permitted widescale screening of sera (Black, 1962; Rosen, 1962). The technique also enabled elucidation of the antibody response following recovery from the natural disease (Black and Rosen, 1962; Krugman, Giles, Friedman and Stone, 1965) and following vaccination (Krugman, Giles, Jacobs and Friedman, 1963; Fulginiti and Kempe, 1965; Watson, 1967).

The sensitivity of the neutralization test rests, *inter alia*, on an efficient host cell system and the use of an adequate amount of accurately pre-determined virus inoculum. Cell lines such as the Hep-2 (Mcrumb, Kress, Saunders, Snyder, Schluederberg and Baltimore, 1961; Black and Rosen, 1962), human amnion (Kunita *et al.*, 1963; Mares, Drevo, Starck and Zavadova, 1966) and Lu 106 (Norrby, 1967) which support rapid growth of the virus are preferred because frequent changes of the maintenance medium are avoided.

Although the infectivity of a culture of measles virus can be reasonably accurately determined by end-point titrations (Underwood, 1959; Yarosh and Armstrong, 1963) and the virus pool can be preserved in a deep-frozen state at -70°C for several months without significant loss of titre, (Norrby, 1967), the results of a test stemming from the use of such a virus preparation can be influenced by the ratio of the concentration of

infectious to non-infectious virus particles present in the sample (Mayer, Brooks, Douglas and Rogers, 1962). The phenomenon of interference or blockade of the reaction between antibodies and infective virus particles by non-infectious virus and its antigens has been fully investigated in an experimental poliovirus system by Gard (1957) and Melen (1960) and in measles by Drevo and Mares (1967). The elegant experiments of the latter clearly demonstrated that the antibody titre was a function of the dose of the antigen. Nevertheless, considerable reduction in titres occurred when preparations having optimal amounts of infectious virus but higher amounts of non-infectious antigens were used. Meyer et al. (1962) observed that low virus doses of the order of 2 to 6 TCD_{50} per 0.1 ml as well as high doses of the order of 6,000 TCD_{50} per 0.1 ml did not yield reproducible results and they found that the relation between virus dose and antibody titre was different from that within the accepted limits of the commonly used antigen doses. Both Meyer et al. (1962) and Drevo and Mares (1967) obtained a two-fold change of the antibody titre by changing the virus dose by 0.5 log_{50} units.

The amount of virus used varied from 100 to 1000 TCD_{50} per 0.1 ml (Mcrumb et al., 1961a; Black and Rosen, 1962; Mares et al., 1966) but about 100 TCD_{50} per 0.1 ml were recommended (Meyer et al., 1962).

There has been no uniformity of procedure regarding

the time and temperature of incubation of the serum-virus mixtures. Toyoshima et al. (1959) used 60 minutes incubation in an ice bath. De Meio and Gower (1961), Meyer et al. (1962) and Cutchins (1962) used one hour at 4°C. Mutai (1959) and Imagawa and Adams (1963) used 30 minutes at room temperature and then 30 minutes at 4°C. Norrby, Lagercrantz, Gard and Carlstrom (1963) exposed the mixtures to one hour at room temperature followed by rrfrigeration overnight. Kunita et al. (1963) found one hour at room temperature to be adequate whereas Ya Shushi (1964) used 3 hours at 37°C.

Drevo and Mares (1967) who re-investigated the problem in detail, showed that the speed of the virus inactivation was time and temperature-dependent. It was slowest at 5°C and most rapid at 37°C. Titre values increased linearly with prolongation of the incubation period and with increasing temperature. Because thermo-inactivation of the virus was a complicating factor in the assessment of the true antibody titres, they determined the half-time of measles virus thermo-inactivation. The values given were 22, 10 and 3 hours respectively at 5, 25 and 37°C (Drevo and Mares, 1967). On the basis of reproducible data obtained over a period of three years, they recommended an incubation time of 4 hours at 25°C in the standard neutralization test (Drevo and Mares, 1967).

A simple method of obtaining samples having

relatively high concentrations of infective virus was to harvest the material early during the phase of active virus replication in a fast growing system (Norrby et al., 1965b). A point of additional importance for the reproducability of results was to fix a specific period after inoculation for the final reading. The haemo-adsorption test increased the objectivity of the readings but it has been ignored or not adequately stressed in most studies (Norrby, 1967).

Rinderpest: The in vivo neutralization test using natural and experimental hosts to detect rinderpest antibodies is now only a matter of historic importance. The successful propagation of rinderpest virus in cell culture (Plowright and Ferris, 1957) has simplified the procedure for recognition and quantitation of antibodies developing after natural exposure or after vaccination (Plowright and Ferris, 1962a).

Semmer (1893) is reputed to have first recognized the protective value of serum and milk from cattle recovering from rinderpest (Curasson, 1942). The first in vivo neutralization test for rinderpest was carried out by Nakamura (1940). He demonstrated antibodies in rabbit anti-rinderpest sera by inoculating pre-incubated serum-virus mixtures into rabbits. Likewise, Walker, Baker and Jenkins (1946) demonstrated antibodies in cattle by mixing dilutions of the virulent virus with undiluted serum and inoculating the mixture after

incubation for two hours in the refrigerator into susceptible calves. Shope and Griffiths (1946) used the same technique to detect antibodies to rinderpest in the sera of chicks that hatched from infected fowl embryos. Jenkins and Walker (1946) recognized antibodies in cattle convalescent of rinderpest by inoculating serum-virus mixtures into rabbits. They used a strain of lapinized-rinderpest virus which was capable of inducing only fever in inoculated rabbits. Absence of fever in the experimental rabbits was taken as evidence of neutralization. The serum constant-virus dilution technique was employed.

The detailed studies of Scott and Brown (1958), Brown and Scott (1959), Huard, Andre and Fournier (1959) on the rabbit neutralization test were based on Nakamura's (1940) original observations. Scott and Brown (1958) used the serum dilution-virus constant technique. Inactivated acute and convalescent phase sera were prepared in five- or ten-fold serial dilutions and mixed with equal volumes of a pre-tested stock suspension of lapinized virus containing 20 to 200 ID_{50}. The mixtures were held either for one hour at 37°C or for 20 hours at 4-10°C. Each mixture was then inoculated intravenously into five rabbits which were killed five days later and examined for virus-specific lesions. The duration and temperature of incubation did not significantly influence the antibody titres, but

the dose of virus did; a ten-fold increase in virus reduced the antibody value by 1-0 to 1-6 log units. Later Brown and Scott (1959) tried to simplify the technique and make it economical for screening sera of cattle for antibodies. Sera from three cattle were pooled and incubated with pre-determined amounts of virus and inoculated into two rabbits. If neither rabbit reacted, the sera were tested individually to determine which contained antibodies.

Huard et al. (1959) employed the virus dilution-serum constant method requiring 8 to 16 rabbits per sample. A difference of 100-fold or greater between the titres of the virus neutralized by acute and convalescent phase sera was regarded as proof of neutralizing antibody. In spite of its extravagance the rabbit neutralization test proved valuable at a time when no alternative technique was available (Scott, 1964).

A neutralization test in which fowl embryos were used as indicator hosts and in which the avianized strain of rinderpest virus was used as the source of antigen was developed and described by Furutani, Nakamura, Ishii and Kurata (1954), Nakamura, Kishii, Kiuchi and Resinger (1955) and Nakamura (1957b). Evidence of neutralization was indirect and depended on the examination of the pooled spleens of the inoculated embryos for complement-fixing activity, a rather cumbersome procedure. Piercy, Scott and Witcomb (1958), on

the other hand, relied on embryo mortality as the criterion of free virus in the serum-virus mixtures. They employed the BA strain of virus which, in their hands, was known for its high specific lethality. There was quantitative agreement between the results obtained by this technique and by the rabbit neutralization test.

Imagawa (1965) used a neurotropic, mouse-adapted strain of rinderpest virus in a neutralization test with newborn mice as indicator hosts. Serum-virus mixtures were inoculated intracerebrally and symptoms of paralysis and death were taken as evidence of infection. Parallel titrations in infant mice brain and cell cultures revealed that the antibody titres from the *in vivo* test were two to four-fold less than from the *in vitro* test.

Plowright and Ferris (1961b) developed qualitative and quantitative cell culture neutralization tests. The former was a screening test for determining the susceptibility of experimental cattle to rinderpest. Undiluted and unheated sera were mixed with 60 to 600 TCD_{50} of virus, held overnight at $4^{o}C$ and were tested for the presence of free virus in two tubes of calf kidney cells. The cultures were examined for up to 12 days for cytopathic changes. A similar test was described by Johnson (1962) but he inactivated the sera at $56^{o}C$ for one hour. Plowright and Herniman (cited by Plowright, 1968) did not find any significant differences

in the titres of standard sera tested before and after inactivation at 56°C for 30 minutes.

In their quantitative serum-virus neutralization test, Plowright and Ferris (1961b) used the serum dilution-virus constant method whereas Johnson (1962) favoured the virus dilution-serum constant method using inactivated serum. The time and temperature of incubation of virus-serum mixtures was about 18 hours at 4°C (Plowright and Ferris, 1961b; Plowright, 1962b). More recently, Plowright and Herniman (Plowright, 1968) observed that the virus-antibody reaction was almost instantaneous at 4 or 37°C but the union was unstable for about 24 hours at 4°C and 4 hours at 37°C. Dilution entailed some degree of dissociation. The antigen-antibody complex was relatively stable after one hour at 37°C followed by 18 to 24 hours at 4°C in phosphate buffer saline at pH 7.2 (Plowright, 1968).

An inverse relationship between the dose of the virus and the titre of neutralizing antibodies existed (Plowright and Ferris, 1961b). An increase of one $\log_{10}$ in the dose of virus entailed, on an average, a reduction of 0.5 to 0.6 $\log_{10}$ units in the SN_{50} titre of the standard serum.

Canine distemper: The first neutralization test for distemper used the dog as the indicator host (Laidlaw and Dunkin, 1928b). A mixture of 11 ml of canine hyperimmune distemper serum and 0.25 ml of the stock

suspension of virulent virus was held at 37°C for one hour and then inoculated subcutaneously into a dog. A control dog was inoculated simultaneously with the stock suspensions of virus without serum. The latter developed mild clinical signs of distemper and recovered. The test dog withstood challenge infection with virulent virus 21 days later. It was not stated whether the control dog was also retested with virulent virus.

Earlier, Laidlaw and Dunkin (1928a) noted that serum from recovered ferrets had no neutralizing effect on the virulent virus and they concluded that immunity in the ferret was cellular and not humoral. Cabasso and Cox (1952), on the other hand, successfully demonstrated neutralizing antibodies in ferret sera in their studies on the adaptation to fowl embryos of a wild strain of distemper virus isolated from a case of canine encephalitis. In its seventh serial passage on the chorioallantois of the fowl embryo, the strain was still pathogenic to the ferret. Distemper-immune ferret serum, two samples of immune canine serum, and a commercial anti-"hard pad disease" serum were mixed in 4 ml amounts with 1.0 ml of a 40 per cent suspension of chorioallantoic membranes infected with the encephalitis strain and incubated at room temperature for 60 to 90 minutes and then inoculated into batches of two ferrets per sample. While the control ferrets died of distemper, the test ferrets remained well. Re-

inoculation with virulent distemper virus 21 days later showed that they were fully susceptible indicating that there was no free virus in the serum-virus mixtures used in the cross-neutralization test.

The development of the *in ovo* neutralization test (Cabasso and Cox, 1949; Cabasso, 1951) was a logical outcome of the successful adaptation of distemper virus to the developing fowl embryo (Haig, 1948; Cabasso and Cox, 1949). The test was a boon to the pharmaceutical industry and earned pride of place in distemper serology. The fowl embryo was a convenient inexpensive experimental host and the results of the test were fairly clear cut. Embryos inoculated with virus alone or virus mixed with normal ferret, canine and guinea pig serum showed well-defined or confluent plaques in the chorioallantoic membrane, the pathognomonic lesion of the egg-adapted virus, whereas embryos receiving immune serum-virus mixture developed no abnormalities (Cabasso and Cox, 1949).

Although tedious, the test was a valuable tool in studies of the immunogenesis of natural and experimental distemper in the dog (Ott *et al.*, 1955; Crook, 1957; Rockborn, 1957b), in assessing the immunizing efficiency of vaccines (Baker, *et al.*, 1954; Burgher, Baker, Sarkar, Marshall and Gillespie, 1958; Gillespie *et al.*, 1958), and in demonstrating serological relationships between different strains of distemper

virus (Belcher, 1951; Gillespie, 1956). The specificity of the test was unequivocally proved by the elegant and elaborate studies of Gillespie et al. (1958) and Karzon et al. (1961). A standard dose of 100 to 300 ID_{50} of virus was mixed with equal volumes of diminishing five-fold dilutions of serum held at 5°C overnight. Then 0.2 or 0.1 ml of the mixtures were inoculated on to the chorioallantoic membranes of 7 to 8 day old embryos by Gorham's method (Gorham, 1957a). Simultaneously virus titrations consisting of normal serum mixed with equal amounts of virus were carried out (Gillespie et al., 1958; Gorham, 1960). The embryos were examined seven days later for virus-specific lesions.

Baker et al. (1954) noted that a ten-fold increase in the dose of virus decreased the antibody titre eight-fold. Karzon (1955), Gillespie et al. (1958) and Karson et al. (1961) found that the neutralization reaction followed first-order kinetics with a linear slope of approximately one.

Using standard sera of canine origin Schindler (1954) found good correlation between the results of embryo neutralization tests and protection experiments in dogs using antiserum. Imagawa et al. (1954) and Karzon (1955) used a constant serum-virus dilution technique and claimed to have obtained reproducible results. However, most workers have favoured the use of constant amounts of virus and falling dilutions of the

serum (Gorham, 1960).

To date no well-documented reports of successful distemper neutralization in cell cultures are available. The dog-kidney adapted strains produced recognizable cytopathic effects but the end-point titrations were difficult to estimate without microscopic examination of stained preparations of the cultures; a fact not adequately emphasized in any published report. Fowl embryo cell culture-adapted strains produce a non-spectacular cytopathic effect consisting of rounding and necrosis of the cells leading to the disintegration of the cell sheet (Karzon and Bussell, 1959; Prydie, 1968). The effect was not only dose-dependent but was also influenced by environmental conditions (Warren, 1960; Prydie, 1968). With diluted inocula the cytopathic effect was slow in onset and progress and was often indistinguishable from the changes associated with ageing of the cells.

Rockborn, Norrby and Lannek (1965) carried out a neutralization test in primary dog kidney cell cultures using 100 TCD_{50} per 0.1 ml of the Rockborn strain of distemper virus and two-fold dilutions of sera from dogs immunized with a commercial vaccine. They did not define the parameters of the test nor did they comment on the value of the test in comparison with the other tests.

Sucking mice inoculated with mouse-adapted strains

of distemper virus developed overt neurological signs ending in death (Arakawa, Muto, Kaneko and Seki, 1959; Carlstrom, 1959a). Virus titres in mice brains were comparable to the titres in fowl embryos and a neutralization test was developed to detect distemper virus strains (Carlstrom, 1959a; Burnstein et al., 1958) and to differentiate them serologically from similar mouse-adapted strains of measles and rinderpest viruses (Imagawa, 1965, 1968).

OBJECTIVES

The objective of the present study was to elucidate the nature and degree of the antigenic relationships between the viruses of measles, rinderpest and distemper. The approach was largely serological. A restricted study using a small group of young puppies born of a distemper-vaccinated bitch was undertaken with the primary objective of determining the antibody response to measles and distemper vaccines in the presence of maternally-derived distemper antibodies.

The measles haemagglutination-inhibition test is a recent development in measles serology. The application of the test to rinderpest sero-diagnosis is even more recent and relatively little is known of its potential use in distemper diagnosis. I have examined in detail the parameters of the measles haemagglutination-inhibition reaction in order to devise a simple, workable procedure for the detection of rinderpest and distemper antibodies.

The complement-fixation and immuno-diffusion tests using measles and distemper-infected tissues and cell cultures were studied to delineate the parameters of the reactions in the homologous systems and to determine the nature and magnitude of cross-reactions.

Studies on the direct comparison of the "medipest" viruses by testing the virus preparations against the same samples of sera derived from their natural hosts or

from a common experimental host are limited. Furthermore, in most studies assessment of the neutralizing ability of convalescent sera in respect of the three viruses has not been made on comparable host systems; rinderpest and measles sera were examined for neutralizing activity in the virus neutralization test in tissue culture, whereas in distemper the in ovo neutralization test has been the most extensively used system. In the present investigation an attempt was made to bridge the gap by performing the virus neutralization tests under comparable conditions.

Finally, the behaviour of measles and distemper viruses in alien hosts was studied to ascertain first the nature of the antibody response to primary inoculation and reinoculation with live and inactivated virus and, secondly, the nature of the anamnestic responses to live and inactivated heterologous viruses.

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VIRUS ISOLATION

MEASLES

Measles virus was isolated in primary patas monkey kidney (PMK) and Vero cells from a specimen of human liver tissue collected from a fatal "clinical" case of measles and forwarded by Dr. Margaret Haire, Department of Medical Microbiology, Queen's University, Belfast. Aetiological confirmation that the isolated agent was measles virus stemmed from haemadsorption-inhibition and virus-serum neutralisation tests and the demonstration of haemadsorption, specific haemagglutinins and complement-fixation antigens in the infected cells. The strain was designated the Belfast strain.

CANINE DISTEMPER

The virus was isolated in primary canine kidney (PCK) cells from lung specimens of three out of nine alleged clinical cases of canine distemper. One of the isolates was named the CTVM strain and was identified as canine distemper virus by neutralisation and complement-fixation tests.

VIRUS STRAINS

MEASLES

Three cell culture-adapted strains were used. One with a passage history, PH-26 V_3 A_4, was received from Dr. F. T. Perkins, Medical Research Council Laboratories, Holly Hill, Hampstead, and was designated the Holly Hill strain. The strain was maintained in FL cells. The second was a Swedish strain and was supplied by Dr. S. Gardner, Virus Reference Laboratories, Colindale. The strain was initially maintained in LU106 cells in the Karolinska Institute Laboratories, Stockholm and had undergone one passage in W1-38 cells at Colindale. Following receipt, the virus underwent six additional passages in W1-38 cells and was later propagated serially in FL cells.

The Beckenham strain was a commercial live measles vaccine with the trade name "Wellcovax". According to the manufacturers,[1] the vaccine strain was derived from Ender's original Edmonston B strain (Enders et al. 1960). After four serial passages in PMK cells, the strain was maintained in FL cells.

CANINE DISTEMPER

The virulent CTVM strain was passaged seven times in PCK cells and adapted to MDCK cells.

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1. Wellcome Research Laboratories, Beckenham.

The Rockborn and the Onderstepoort strains of distemper virus were obtained as commercial vaccines from Hoescht Pharmaceuticals Limited, Bradford and Glaxo Laboratories Limited, Greenford, respectively. The Rockborn strain was propagated serially in MDCK cells, whereas the Onderstepoort strain was maintained in first or second generation cultures of fowl embryo fibroblasts (FEFC).

ELECTRON MICROSCOPY

The identity of the viruses used was confirmed morphologically by electron microscopy (Figs. 1-4).

Electron Microscope: The electron miscroscope used was the AEI-EM-6B[1].

Stain: Phosphotungstic acid[2] used for negative staining was prepared by dissolving 1.5 grams in 100 ml. of de-ionised distilled water. The pH was adjusted to 7.2 by the slow addition of drops of 1N potassium hydroxide.

Method: Pellets of infected cell cultures and tissue suspensions were prepared by high speed centrifugation in the cold. Two drops of the stain were added to a pellet which was disintegrated by stirring for approximately 30 seconds. The gross debris was removed and discarded. A carbon-coated collodion grid was gently

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1. Associated Electrical Indistries Ltd., Harlow, Essex.
2. George T. Gurr Ltd., London.

lowered face downwards on to the surface of the stain. After 30 seconds the grid was lifted, excess stain was removed by blotting and the grid was left to dry overnight in a desiccator containing calcium chloride. The grid was then screened in the electron microscope. Good intact fields were searched for at an initial magnification of 1,500 and then scanned for the presence of virus at higher magnifications.

CELL CULTURES

Primary human amnion monolayer cultures were supplied by the City Hospital, Edinburgh. Primary patas and cynomologous monkey kidney cells were obtained as suspensions from Wellcome Laboratories, Beckenham.

PREPARATION OF PRIMARY CULTURES

Kidney Cells: Trypsinized monolayer cultures of calf, ferret, guinea pig, hamster, kitten, mouse, puppy, rabbit and rat kidneys were prepared by a modification of the multiple extraction procedure described by Youngner (1954). Excised kidneys were washed three times in Earle's[1] or Hank's[2] balanced salt solution (BSS) pre-warmed to 37°C, and containing 500 units, 500 ug and 20 ug per ml. respectively of penicillin,[3] streptomycin sulphate[3] and nystatin[4] or fungizone.[4] The kidneys were transferred to a Petri dish and minced with scissors after removing the capsule and pelvis. The mince was washed thrice in Earle's or Hank's BSS and transferred to a trypsinization flask. One hundred ml of an 0.05 per cent trypsin[5] solution in phosphate buffered saline (PBS)

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1. Earle, 1943.
2. The Oxoid Division of Oxo Ltd., London.
3. Glaxo Laboratories Ltd., Greenford.
4. E. R. Squibb & Sons, Speke, Liverpool.
5. Difco Laboratories, Inc., Detroit, U.S.A.

(pH 7.2) were added and the suspension was stirred for 30 minutes at room temperature (20 to 22°C). The tissue fragments were allowed to settle and the supernatant fluid was discarded. One hundred ml of fresh 0.05 per cent trypsin solution were added and the suspension was stirred at a rate just short of bubbling for 45 minutes at room temperature. The trypsinization was repeated twice and the harvests between the cycles were stored on ice in a flask containing 50 ml of outgrowth medium supplemented with 10 per cent inactivated calf serum and an antibiotic mixture comprising penicillin, streptomycin and nystatin or fungizone. The remnants of tissue were then subjected to overnight trypsinzation (Bodian, 1956) at 4°C using 100 ml of 0.05 per cent trypsin solution.

The cell suspensions were filtered through four layers of sterile gauze and the cells packed by horizontal centrifugation at 600 r.p.m. for 15 minutes. The cell pellets were suspended in 100 ml of outgrowth medium and sedimented again by centrifugation at 800-1000 r.p.m. for 10 minutes. The cells were dispensed into suitable culture vessels after resuspending 1:150 to 1:200 by volume in outgrowth medium.

Bovine Foetal Lung Cells: Cultures of foetal calf lung fibroblasts were prepared by a single cycle of trypsinization at room temperature for one to two hours with 0.2 per cent trypsin solution. If necessary, the residual tissue was stirred overnight at 4°C with 0.05

per cent trypsin, or 0.03 per cent versene.

Canine Lung Cells: Canine lung fibroblasts and canine lung alveolar macrophage cultures were prepared by the procedures described by Appel and Jones (1967) and Myrvik (1961) respectively. However, in the light of the observation of McKay (1969) that sheep lung alveolar macrophages sometimes failed to attach to glass surface in the presence of antibiotics, suspensions of canine lung alveolar macrophages were prepared in outgrowth media containing no antibiotics and were dispersed into plastic Petri dishes planted with cover-slips and incubated at 37°C for two hours in a humid atmosphere of 5 per cent carbondioxide. The medium was then aspirated and replenished with outgrowth medium containing penicillin (100 units/ml), neomycin[1] (100 ug/ml), Kanamycin[2] (199 ug/ml), Polymyxin,[3] Bacitracin[2] and fungizone (25 ug/ml), and incubated at 37°C in a humid atmosphere of 5 per cent carbon-dioxide.

Canine Spleen Cells: Puppies, 4 to 10 days old, were destroyed with intravenous pentobarbitone[1] and exsanguinated. Their spleens were excised with sterile precautions and minced with scissors. The mince was transferred to a flask and stirred in medium-199[3]

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1. Boots Pure Drug Co. Ltd., Nottingham.
2. The Bayer Products Company, Surbiton.
3. Burroughs Wellcome & Co., London.

containing 0.5 per cent lactalbumin hydrolysate for one hour at room temperature. The tissue fragments were then suspended in 25 ml of Hank's BSS and centrifuged at 1000 r.p.m. for five minutes. The sediment was re-suspended in 50 ml of an 0.01 per cent versene solution in PBS and stirred at room temperature for one hour.

The cell suspensions were pooled and then centrifuged in 25 ml volumes at 1000 r.p.m. for five minutes. The cell pellets were resuspended 1:50 by volume in outgrowth medium and dispersed into plastic Petri dishes with cover-slips in 5 ml amounts. The dishes were incubated in a humid atmosphere of 5 per cent CO_2.

Canine Testes Cells: Testes stripped of their tunica vaginalis were minced with scissors and the mince was subjected to a multiple extraction procedure of trypsinization at room temperature with 0.25 per cent trypsin solution. Each cycle lasted 15-20 minutes and five harvests were made. Between harvests the suspensions were stored on ice in the presence of outgrowth medium. The sedimentation and dispersion of the cells were similar to those described for mammalian kidney cell cultures.

Fowl Embryo Fibroblasts: Nine to 10 days' old fowl embryos from White Leghorn or Rhode Island Red hen eggs were used for the preparation of fowl embryo cell cultures. However, in the study of the influence of the age of the fowl embryo, the source of fibroblasts,

on the onset of the CPE induced by the Onderstepoort strain of canine distemper virus, the age of the embryos varied from 5 to 18 days.

Embryos were collected aseptically, decapitated and freed of entrails and limbs and minced with scissors in a Petri dish. The mince was washed thrice with Hank's BSS and once with 0.1 per cent trypsin solution. The tissues were then subjected to a single cycle of trypsinization at room temperature for one hour.

Leukocyte Cultures: The general procedure was similar to the one described for the cultivation of rinderpest virus strains in bovine leukocytes by Tokuda et al. (1962).

Cattle and sheep inoculated subcutaneously with different doses of the Holly Hill strain of measles virus were bled from the jugular vein at different post-inoculation intervals. Similarly, rabbits inoculated intravenously with the virus were bled from an ear vein. The blood was treated with one-twentieth volume of 0.2 per cent solution of heparin[1] in PBS to which the antibiotic mixture of penicillin (1000 units/ml), streptomycin (1000 ug/ml) and fungizone (40 ug/ml) was previously added. The whole blood was dispensed into culture tubes and glass Petri dishes containing coverslips in 3 ml and 8 ml amounts respectively. Tube cultures were incubated in a stationary state

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1. Boots Pure Drug Co. Ltd., Nottingham.

aerobically at 37°C whereas Petri dish cultures were held in a humid atmosphere of 5 per cent CO_2 at the same temperature.

Leukocyte Suspensions: Cattle, sheep and rabbits inoculated with measles virus were bled and the leukocytes were separated from whole blood by the procedure originally devised by Amos and Peacocke (1963) and later modified by Aub, Sanford and Li-Hsia Wang (1965). Twenty ml of blood were drawn into bottles containing 1 ml of a 10 per cent solution of disodium EDTA[1] and 1 ml of a 10 per cent solution of polyvinyl pyrollidone.[1] The salts were dissolved in PBS containing penicillin (1000 units/ml), streptomycin (1000 ug/ml) and fungizone (40 ug/ml). The erythrocytes were allowed to sediment for 30 to 45 minutes and the supernatant plasma was centrifuged at 500 r.p.m. for 10 minutes. The packed cells were resuspended in 1.5 ml of the supernatant plasma. The leukocyte suspensions were overlaid on LLC-MK2, FL or Hep-2 cell monolayers in 0.1 ml amounts and incubated at 37°C.

CELL LINES

LLC-MK2, Bsc.1, FL, AV_3 and MDCK cells were obtained from Flow Laboratories, Limited. Hep-2 cells were supplied by the Department of Bacteriology,

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1. The British Drug House, Ltd., Poole.

Edinburgh University Medical School and HeLa cells by the Moredun Institute, Gilmerton. Vero and W1-38 cells were obtained from the Virus Reference Laboratories, Colindale.

Sub-cultivation of Cell Cultures: The media in the culture vessels were discarded and the monolayers were washed thrice with Hank's BSS. For detaching the cells from the glass surface an 0.25 per cent solution of trypsin or 0.03 per cent solution of EDTA in PBS were used. MDCK cells required 10-15 minutes treatment with the trypsin solution but with most cell lines the trypsin was allowed to act for one minute and the discarded. The culture vessels were then incubated in an inverted position at room temperature or 37°C for 10-15 minutes. About 20 ml of outgrowth medium were added and the cells were detached easily by gentle agitation. Pipetting of the suspension ensured adequate dispersal of the cells. The suspension was then centrifuged at 1000 r.p.m. for five minutes.

When versene solution was used the monolayers were incubated with the reagent for 15 to 30 minutes at 37°C. Cell clumps were dispersed by gentle pipetting and the resulting suspension was sedimented.

The packed cells were dispensed into culture vessels after diluting 1:100 to 1:150 by volume.

CELL CULTURE MEDIA

Outgrowth media used for the cultivation of most primary cell cultures and established mammalian cell lines contained Eagle's minimum essential medium (M.E.M.)[1] or medium 199 as the base supplemented with 10 per cent heat-inactivated commercial calf serum obtained from different sources.[2] Penicillin, streptomycin and nystatin or fungizone to a concentration of 500 units, 500 ug and 20 ug respectively per ml and sodium bicarbonate to a final concentration of 0.35 per cent were also incorporated. However, the serum requirements of certain cell types varied from 20 to 40 per cent (Tables 1 and 2). Rabbit cultures grew better in the presence of rabbit serum, although they were maintained satisfactorily with calf serum. Furthermore, for the propagation of canine distemper virus strains canine primary cell cultures and MDCK cells were grown and maintained on media containing foetal or colostrum-deprived calf serum.

Maintenance media comprised Earle's BSS enriched with lactalbumin hydrolysate[3] (0.5 per cent) and yeast

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1. Burroughs Wellcome & Co., London.
2. Flow Laboratories Ltd., Irvine; The Oxoid Division of Oxo Ltd., London; Burroughs Wellcome & Co., London.
3. V. A. Howe & Co. Ltd., London.

extract[1] (1 per cent) (E.Y.L.) or medium 199 as the base supplemented with 2 to 5 per cent calf or foetal calf serum. A higher percentage of serum was required to maintain leukocyte cultures and dog spleen cells (Table).

Fowl embryo fibroblast cultures were initially grown on medium 199 containing 4 per cent calf or horse serum and maintained on E.Y.L. or medium 199 containing no serum.

In general, the bicarbonate concentration in maintenance media was kept at 1.10 per cent.

PRESERVATION OF CELL LINES

The method recommended by Dougherty (1962) was used with slight modifications to preserve cell lines. Four volumes of trypsin or versene-dispersed cells (10^{8} to 9 per ml) were added to 1 volume of a mixture of equal parts of dimethyl sulphoxide[2] (DMSO) and foetal calf serum. The mixture was cooled step-wise to freezing point in about two hours. The frozen cultures were then transferred to the deep-freeze cabinet ($-75^{\circ}C$).

STAINING

Cover-slip cultures were rinsed in PBS and fixed in

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1. Difco Laboratories Inc., Detroit
2. Associated Electrical Industries Ltd., Harlow, Essex.

Bouin's fluid (Paul, 1959) or in methanol containing 5 per cent glacial acetic acid for 5-10 minutes and stained as a routine in duplicate or triplicate with haematoxylin-eosin and giemsa stains (Paul, 1959). Phloxine and tartarazine (Lillie, 1954) and acridine orange (Harris, 1964) methods of staining were applied for confirming the presence of inclusion bodies.

PRODUCTION OF VIRUS STOCK POOLS

Measles: Large flasks were seeded with a high concentration of FL cells such that confluent monolayers developed in four to six days. The monolayers were washed thrice with Hank's BSS or PBS after discarding the cell culture fluids and were seeded with 10-20 ml of a 1:10 dilution of the virulent Belfast or the three adapted strains of measles virus. Adsorption proceeded for two hours at room temperature. The unadsorbed virus was decanted and the monolayers were washed two to three times with Hank's BSS or PBS. After the addition of maintenance medium the bottles were incubated aerobically at 37°C.

At the height of the CPE the cultures were frozen and thawed thrice with the fluids. The harvests were centrifuged at 1000 r.p.m. for 15 minutes to remove cell debris. Sodium bicarbonate solution and inactivated calf serum were added to the supernatant fluids to give final concentrations respectively of 0.2 and 10 per cent. The fluids were distributed into 5 ml aliquots and stored at -75°C.

Canine Distemper: Flask cultures of MDCK cells were dispersed with an 0.25 per cent trypsin solution and the cells washed twice to remove the trypsin. The cells were suspended 1:5 by volume in outgrowth medium and distributed into small bottles in 2 ml amounts. To one

set of bottles undiluted CTVM strain of canine distemper virus was added in 2 ml volumes. To the second set of bottles a similar volume of the Rockborn strain of distemper virus was added. The suspensions were held at room temperature for one hour and then seeded into flasks mixed with about 80 to 100 ml of outgrowth medium.

At the height of the CPE when more than 50 per cent of the cells were involved, the cultures were frozen and thawed. The procedure for clarification and preservation of the culture fluids was similar to that for measles virus except that foetal calf serum was used in the place of calf serum.

VIRUS ASSAY

Measles: Infectivity titres of the strains of virus propagated in different cell cultures were estimated in FL cells. The test pool was diluted in medium 199 containing 5 per cent calf serum in ten-fold series in 1 ml amounts. At least five tube cultures were inoculated with each dilution of the virus in 0.1 ml amounts. Adsorption was allowed to proceed at room temperature for two hours or at 37°C for one hour. The unadsorbed virus was discarded and the cultures were washed with PBS or Hank's BSS 2-3 times and then replenished with maintenance medium. On the 14th day post-inoculation, the cultures were tested for haemadsorption of simian erythrocytes and the results were

expressed as 50 per cent end-point doses per ml (TCD_{50}/ml) as calculated by the method of Reed and Muench (1938).

Canine Distemper: Titrations were carried out in two to three days' old MDCK tube cultures with cover-slips. Medium 199 containing 5 per cent foetal calf serum was used as diluent. On the 14th day post-inoculation visual assessment of the CPE was confirmed by staining cover-slip cultures.

VIRUS GROWTH CURVE STUDIES

Measles: Three days' old PMK cells were infected respectively with $10^{4.6}$ and $10^{4.8}$ TCD_{50} per ml of the Belfast and the Holly Hill strains of measles virus. FL cultures of the same age were infected with similar doses of the two strains. At intervals of two days, five tube cultures from each group were frozen and thawed thrice and the fluids pooled into small bottles. The pools were divided into two, 9 volumes of one half being mixed with 1 volume of inactivated calf serum and stored at -75°C and the second half being stored at -75°C without the addition of serum. The former were used to estimate infectivity titres and the latter the titres of haemagglutinins and CF antigens.

ACTINOMYCIN-D TREATMENT

Measles: The effect of actinomycin-D[1] on the replication of measles virus was investigated by incubating two sets of 4 days' old monolayer cultures of FL cells at 37°C for four hours with 1 ml of maintenance medium containing respectively 0.05 and 0.1 ug of the drug. The treated cells were then washed thrice with maintenance medium and then seeded with 0.1 ml of $10^{4.6}$ TCD_{50} of the virus. Simultaneously, a third set of untreated cultures were inoculated with the same amount of virus. After an adsorption period of two hours at room temperature the unadsorbed virus was discarded and the monolayers were washed 2-3 times with Hank's BSS and then replenished with maintenance medium. From the second day post-infection, three tubes from each group of the treated cultures and three from the untreated groups were removed and subjected to three cycles of freezing and thawing. Harvests were made on alternate days until the 18th day post-inoculation. The fluids from replicate tubes were pooled and stored separately in 2 ml aliquots for virus, haemagglutinin and CF antigen titrations.

In another study, attempts were made to adapt the Belfast strain of measles virus to actinomycin-D-treated MDCK cells. Five passages were made in treated cells.

Canine Distemper: The first study was designed to

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1. Merck Sharp and Dohme Ltd., Hoddesdon, Herts.

determine the effect of concentrations of the drug on the onset of the CPE induced by the CTVM and Rockborn strains of canine distemper virus in MDCK cells. A batch of 4 days' old tube cultures of MDCK cells with cover-slips were divided into four groups. The first three groups were treated respectively with 0.025, 0.05 and 0.10 ug of the drug per ml for three hours at 37°C. The media were then discarded, the cultures were washed 2-3 times with maintenance medium and infected along with the fourth group of untreated cultures with the CTVM strain of distemper virus using neat inoculum of 0.1 ml amounts. The maintenance medium was renewed after two hours adsorption at room temperature.

Another batch of MDCK cells was similarly treated and infected with the Rockborn strain of distemper virus. The rate of development of the CPE in the treated and untreated groups was compared in the two sets of cultures by examination of unstained and stained cultures.

In a second study, the effect of actinomycin-D treatment (0.1 ug per ml) on the infectivity titres induced by the CTVM strain was investigated from the 6th to 9th passage of the virus in MDCK cells. At the height of the CPE replicate cultures were frozen and thawed and the pooled fluids were titrated in PCK cells. The titres were compared with the titres of the virus produced in untreated cells.

In a third study, the influence of the drug on the

onset of the CPE and CF antigen titres in fowl embryo fibroblasts produced by the Onderstepoort strain of canine distemper virus was investigated. Five different concentrations of the drug were used, namely, 0.01, 0.02, 0.03, 0.05 and 0.1 ug per ml. Replicate cover-slip cultures were stained and examined for the CPE from the 2nd to 6th day post-infection. In the comparative study of the evolution of the CF antigen titres in the treated and untreated cultures, replicate cultures were frozen and thawed at 8, 16, 30, 48, 72 and 120 hours post-inoculation and the pooled harvests titrated for specific CF antigens.

PRODUCTION OF ANTIGENS

CONCENTRATION TECHNIQUES

Measles: Flask cultures of FL cells were infected with 20 ml of $10^{4.8}$ TCD_{50} per ml of virus. Fluids were first harvested when 25 per cent of the cells manifested CPE and were replaced with maintenance media comprising equal volumes of bovine amniotic fluid and 2 per cent foetal calf serum. Harvests thereafter were made daily until the LPE had reached its maximum.

The cell sheets were frozen and thawed or sonicated and the suspension was added to the pooled fluids. The harvest was centrifuged at 3000 r.p.m. for 20 minutes and the supernatant fluid was dialysed in 500 ml amounts against "carbowax" (Polyethylene glycol) at 4°C until the contents were concentrated 50 to 100 times. The concentrated fluid was dialysed against 0.15 M Nacl solution for 18-20 hours at 4°C. A saturated solution of ammonium sulphate was added to obtain a final salt concentration of 35 per cent. Precipitation proceeded at 4°C for 2-4 hours. The precipitate was sedimented by centrifugation at 5000 r.p.m. for 30 minutes at 4°C. The supernatant fluid was subjected to further treatment with ammonium sulphate solution until no more precipitation occurred.

The precipitate was dissolved in distilled water and dialysed against 0.15 M Nacl solution for 18-20 hours at 4°C. Further concentration of the solution was achieved

by forced dialysis against polyvinyl pyrrolidone followed by dialysis against 0.15 M Nacl solution. The preparation contained haemagglutinins, complement-fixation and immuno-diffusion antigens.

Canine Distemper: The Rockborn and Onderstepoort strains of the virus were propagated respectively on 2-3 days' old cultures of MDCK cells and 2 days' old cultures of fowl embryo fibroblasts. The maintenance medium for MDCK cells contained 0.5 to 1 per cent foetal calf serum whereas that for fowl embryo fibroblasts contained no serum. At the height of the CPE the cultures were frozen and thawed together with the fluids or the cell sheets were sonicated. The fluids were concentrated as before. The final preparation contained CF and ID antigens.

MEASLES HAEMAGGLUTININS

Commercially prepared Tween-ether haemagglutinins and heat-inactivated haemagglutinins were obtained respectively from Behringwerke AG and Burroughs Wellcome Laboratories Ltd. Crude preparations of haemagglutinins were prepared from measles-infected cell cultures.

Reaction Specificity: Fluids from cell cultures infected with different strains of measles virus were collected at the height of the CPE and were tested for haemagglutinins. Intra cellular haemagglutinins were sought after cell-disruption by freezing and thawing or sonication.

Reactions were deemed specific when simian crythocytes only were agglutinated and when this haemagglutination was inhibited by anti-measles serum.

Comparisons: In studies designed to relate the titres of haemagglutinins to infectivity and CF antigen titres, cell sheets were frozen and thawed with the fluids. Alternatively, fluids were removed and the cell sheets were frozen and thawed or sonicated in a small amount of Hank's BSS and the treated suspensions mixed with the fluids.

Physical Aspects:

Sonication: Infected cells were dispersed with versene or were scraped from the glass surface. The versene was removed by centrifugation. Cells were suspended in 2-10 ml of chilled Hank's BSS and disintegrated at 10 Kc per minute for varying periods in an ultrasonic disintegrator.[1]

Freezing and thawing: Culture bottles or tubes were plunged into a tray containing solid CO_2 moistened with methylated spirit and the monolayers were allowed to freeze. The cultures were quickly thawed by immersing the bottles in a 37°C water-bath. The cycle was repeated twice.

Released haemagglutinins: Fluids from infected

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1. Ultrasonic Disintegrator, 100 Watt Model. Measuring and Scientific Equipment Ltd., London.

cultures were removed, centrifuged at 3000 r.p.m. for 10 minutes and the supernatant fluids titrated for haemagglutinins.

Cell-associated haemagglutinins: Fluids were centrifuged and any cell sediment was suspended in Hank's BSS to the original volume. The monolayer was rinsed 2-3 times with Hank's BSS and any released cells were removed by centrifugation and added to the first pool. The suspension was returned to the bottle and the monolayers were disrupted by sonication or alternate freezing and thawing. The resulting suspension was clarified by centrifugation at 3000 r.p.m. for 10 minutes. The supernatant fluid was titrated for haemagglutinins.

Thermal inactivation: Aliquots of crude preparations of haemagglutinins, Wellcome heated haemagglutinins, the CTVM and Behringwerke Tween-ether and the CTVM deoxycholate-treated haemagglutinins were stored in small tightly-stoppered bottles at 36, 21, 4 and -10°C. At different storage periods a bottle of each product was tested for haemagglutinin activity, the tests being replicated.

In the experiments designed to determine the thermal decay of the haemagglutinins at higher temperatures, Behringwerke Tween-ether haemagglutinins were distributed in 0.5 to 1 ml amounts in glass tubes, 76 mm long by 12 mm wide, and exposed to different temperatures in

a water-bath. At intervals, the tubes were removed to an ice bath. Then titrations were carried out. The experiments were replicated.

Lyophilization: Batches of Behringwerke Tween-ether haemagglutinins were distributed in 1 ml volumes in ampoules and freeze-dried.[1] The ampoules were sealed *in vacuo*. Five batches of the haemagglutinins were titrated before and after lyophilization. With each batch the test was replicated five times.

Chemical Aspects:

pH inactivation: The effect of pH on measles haemagglutinins was investigated by mixing in equal volumes a preparation of Behringwerke Tween-ether haemagglutinins (3.7 $\log_{10}$ units per ml) with the respective buffer solutions. Glycine-Hcl, acetate-phosphate-borate and carbonate-bicarbonate buffers were used for the low (4-5), intermediate (5-7) and high ranges (8-10) of pH respectively. The pH of the solutions was checked with a pH meter[2] and haemagglutinin-buffer mixtures in the range of pH 4 to 10 were dispensed into small bottles in 0.5 ml amounts and stored at -10°C. Titrations were carried out after different storage intervals.

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1. Virtis Macro-Freeze Mobile Unit. Exal Electronic and X-Ray Applications Ltd., Basingstoke, Hants.
2. Electronics Instruments Ltd., Richmond, Surrey.

Treatment with ether: Batches of the crude preparations of haemagglutinins were mixed with diethyl ether in the ratios of 1:1, 1:2 and 1:4 and the mixtures were shaken mechanically[1] at 4°C for one hour. The residual ether was eliminated by bubbling metrogen through the mixtures. The suspensions were titrated.

Treatment with Tween 80: Several trials were made in which the crude preparation of haemagglutinins were treated in a ratio of 10:1 with different concentrations of polyoxyethylene sorbitan monooleate (Tween 80)[2] in distilled water. The mixtures were shaken mechanically at 4°C for five minutes. The treated suspensions were transferred to Visking cellophane tubes[3] and dialysed against 0.15 M sodium chloride solution at 4°C for 18-20 hours. The suspensions were then titrated.

Tween 80-ether treatment: One ml aliquots of a crude preparation of the CTVM haemagglutinins were treated with Tween 80 to give a final concentration of 5 to 10 mg per ml of the detergent. The treated haemagglutinins were then mixed in different proportions with ether and mechanically agitated for periods ranging from 5 to 20 minutes at either 4, 22 or 37°C. Then mixtures were centrifuged at 3000 r.p.m. for 20 minutes. The

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1. Griffin and George Ltd., London.
2. Honeywill and Stein, Ltd., London.
3. The Scientific Instruments Centre, Ltd., London.

organic solvent phase was discarded and the aqueous phase was aspirated. The precipitate that developed between the two phases was dried in vacuo at 4°C overnight in a desiccator over calcium chloride. The dried precipitate was reconstituted in the aqueous phase from which the residual ether had been expelled by bubbling nitrogen. The suspensions were titrated for haemagglutinins.

Treatment with deoxycholate: The method was similar to that outlined by Norrby (1966). A batch of the crude preparation of haemagglutinins was dialysed against PBS (pH 7.8) and divided into aliquots. A small volume of sodium deoxycholate[1] solution was added to each aliquote to obtain final concentrations ranging from 1 to 250 mg per ml. The suspensions were mechanically agitated at room temperature for five minutes and then dialysed against PBS (ph 7.8) for two hours. The samples were then titrated.

Treatment with oxidising agents: To test the effects of oxidising agents on measles haemagglutinins the method outlined by Norrby (1962b) was followed. Aliquots of Behringwerke Tween-ether haemagglutinins having a titre of 3.7 $\log_{10}$ units per ml were mixed with potassium permanganate, potassium periodate, hydrogen peroxide, or iodine of varying molarities (0.01 to 0.0001 M) and incubated at 37°C for 30 minutes. The

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1. The British Drug House, Ltd., Poole.

mixtures were dialysed against an 0.15 M sodium chloride solution for 16-20 hours at 4°C. The suspensions were then titrated.

Treatment with formalin: In a sighting experiment different concentrations of formalin were prepared in 0.15 M Nacl solution and mixed in a ratio of 1:9 with aliquots of Behringwerke measles Tween-ether haem-agglutinins (4.0 $\log_{10}$ units per ml). The final concentrations of formalin ranged from 0.5 to 10 per cent. The mixtures were incubated at 37°C for one hour and then transferred to an ice bath. After dialysis at 4°C for 18-20 hours against 0.15 M Nacl solution the suspensions were titrated for haemagglutinins.

In another trial aliquots of Behringwerke Tween-ether haemagglutinins were incubated with 5.0, 2.5 or 1.25 per cent solutions of formalin in 0.15 M Nacl for periods ranging from 10 minutes to 24 hours at 37°C. The mixtures were chilled on ice before transferring to dialysis tubes. The suspensions were dialysed against 0.15 M Nacl solution for 24 hours at 4°C and then titrated.

Trypsin-inactivation: Trypsin[1] solutions were prepared in PBS (ph 7.2) and mixed with aliquots of Behringwerke Tween-ether haemagglutinins in a proportion of 1:9 such that the final concentrations of the enzyme

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1. The British Drug House, Ltd., Poole.

ranged from 10 to 1000 mg per ml. The mixtures were incubated in a water bath at 37°C. At intervals of 5 to 90 minutes the containers were removed from the water bath and chilled on ice. Twenty-five units of Soyabean trypsin-inhibitor[1] in 0.1 ml amounts were added to arrest further action of trypsin. Titrations were carried out to determine any fall in haemagglutinin titres.

To study the influence of pH on the rate of trypsin action aliquots of Behringwerke Tween-ether haemagglutinins were mixed in equal volumes with buffer solutions designed to give a pH range of 5 to 10. One volume of trypsin solution was added to 9 volumes of the haemagglutinin suspensions to give a final concentration of 25 mg per ml of the enzyme. The mixtures were held at 37°C and the containers were removed at different intervals of incubation to an ice bath. Soya bean trypsin-inhibitor was added and the mixtures were titrated.

COMPLEMENT-FIXATION ANTIGENS

Measles: For the production of CF antigens FL or LLC-MK2 flask cultures were infected with undiluted inocula. At the height of the CPE the maintenance medium was replaced with medium 199 without serum. After further incubation for 24 hours at 37°C the cultures were frozen

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1. The British Drug House, Ltd., Poole.

and thawed or the cell sheets were sonicated. The pooled harvests were clarified by centrifugation at 3000 r.p.m. for 20 minutes. The fluids were concentrated by forced dialysis against "carbowax" followed by precipitation with ammonium sulphate as described before. The final preparation was inactivated at 60°C for 30 minutes, distributed in 2 ml aliquots and stored at -75°C.

Negative control antigens were prepared similarly from uninfected cell cultures.

Canine Distemper:

Cell culture CF antigens: The procedure for production of CF antigens was the same as that described for the concentration of the infective cell culture fluids. Negative control antigens were prepared from uninfected MDCK and fowl embryo fibroblasts. The two types of antigens were inactivated at 37°C for 30 minutes and small aliquots were stored at -75°C.

Tissue CF antigens: A 25 per cent suspension of distemper infected tissues was made in chilled distilled water. The suspension was allowed to settle and the supernatant fluid was clarified by centrifugation at 3000 r.p.m. for 15 minutes. The fluid was dialysed against 0.15 M Nacl solution for 24 hours at 4°C. Any precipitate formed was removed by centrifugation. The supernatant fluid was precipitated with ammonium sulphate and concentrated by forced dialysis against "carbowax"

as described previously.

Other Methods: To study the comparative rates of recovery of CF antigens from measles-infected cell cultures and distemper-infected tissues, by precipitation with acetone, methanol and benzene, suspensions were first concentrated approximately 50 times by forced dialysis with "carbowax". After dialysis against 0.15 M Nacl solution for a few hours the suspensions were distributed in 10 ml amounts and treated with different proportions of chilled acetone, or methanol. Distemper-infected suspensions were also treated with benzene. Precipitation was allowed to proceed overnight at 4°C. The precipitates were sedimented by centrifugation, dried in vacuo at 4°C in a desiccator over calcium chloride and then dissolved in a small amount of 0.15 M Nacl and dialysed against the same diluent for a few hours at 4°C. Titrations were carried out for CF antigens and the titres compared with those obtained from the original untreated suspensions.

Trials were also carried out with measles cell culture and distemper tissue CF antigens to determine the optimal concentrations of ammonium sulphate that permitted the highest percentage recovery of the antigens. Suspensions in 5-10 ml aliquots were treated with saturated ammonium sulphate solution at 4°C such that the final concentrations of the salt varied from 20 to 80 per cent. Precipitation was

allowed to proceed at 4°C overnight. The precipitates and the supernatant fluids were recovered and dialysed against 0.15 M Nacl solution. Supernatant fluids were first concentrated against "carbowax" and then dialysed against 0.15 M Nacl solution. The CF antigen titres in the suspensions from the precipitates and the supernatant fluids were compared.

IMMUNO-DIFFUSION ANTIGENS

The method of demonstration of measles and distemper cell culture 1D antigens was similar to that used for the demonstration of the cell culture CF antigens. However, in the propagation of the two viruses in cell lines designed for the production of the 1D antigens, serum was omitted from the maintenance medium.

The preparation of distemper 1D tissue antigens was similar to that outlined for the specific CF tissue antigens.

EXPERIMENTAL ANIMALS

RABBITS

Young European rabbits of various breeds were used. They were injected either by the intramuscular or by the intravenous route. They were later bled for serum from an ear vein.

GUINEA PIGS

Two groups of guinea pigs were inoculated intraperitoneally with live and heat-inactivated Holly Hill strain of measles virus. The animals were bled from the heart for sera under pentobarbitone anaesthesia.

CATTLE

Ayrshire and Friesian calves, aged two to four months, and four pregnant cows of the same breeds were used in the studies of the antibody respense to measles antigens. Four calves born of the four cows were tested for the presence of colostrally-derived measles antibodies. Ayrshire calves, two to five months of age, were used in the studies with distemper antigens. All inoculations were given subcutaneously.

SHEEP

The sheep that were inoculated subcutaneously with different doses of live Holly Hill strain of measles virus or Tween-ether haemagglutinins were aged three to

six months and belonged to the Blackface, Cheviot and Suffolk breeds.

DOGS

A Harrier-hound bitch about 18 months of age, and seven of its pups were used in the study designed to determine the homotypic and heterotypic responses to inoculations with measles and distemper antigens.

SERA

MEASLES

Human Sera: Adult sera were obtained from human volunteers of different ages. Children's sera were supplied by Dr. J. A. Dudgeon, The Hospital for Sick Children, London.

Monkey Sera: Rhesus (Macaca mulatta) and cynomologous (Macaca cynomologous) sera were separated from specimens of heparinized blood received from the Department of Anatomy, University of Birmingham and the Department of Bacteriology, University of Manchester.

Sheep Sera: Two specimens of reference anti-measles serum prepared in sheep were supplied by Dr. J. T. Perkins, Medical Research Council Laboratories, Holly Hill, Hampstead.

Other Animal Sera: To study the titres of normal agglutinins for simian erythrocytes and non-specific inhibitors of measles haemagglutination sera from cattle, cats, dogs, ferrets, fowls, goats, guinea pigs, horses, pigs, rabbits, rats, reindeer and sheep were obtained from different sources.

To study the specificity of measles haemagglutination-inhibition test antisera for the classified human and animal myxoviruses were obtained from the Public Health Laboratories, Manchester and Colindale, Professor A. P. Waterson, Department of Virology, St. Thomas's Medical School, London and Dr. D. A. MacMartin,

Veterinary Laboratory, Lasswade.

CANINE DISTEMPER

Dog Sera: Sera from dogs with different backgrounds were received from the Veterinary Schools in Edinburgh, Glasgow, Cambridge and Bristol. Sera were also collected from puppies destroyed for the preparation of primary cell cultures.

Specimens of dog and horse hyperimmune distemper sera were supplied by the Wellcome Research Laboratories, Beckenham.

RINDERPEST

Cattle Sera: Sera were obtained from Hill Zebu cattle inoculated with the virulent bovine Hissar strain of rinderpest virus at the Indian Veterinary Research Institute, Mukteswar-Kumaon. Sera were also obtained from the White Fulani herd belonging to the University of Ibadan, Nigeria. There were 27 dairy cows, aged 2½ to 12 years, and having a history of multiple vaccinations with lapinized and cell culture-adapted rinderpest vaccines. In addition, 27 calves, the progeny of the 27 cows, were bled for serum before and after vaccination with cell culture-adapted or caprinised rinderpest vaccine.

Rinderpest hyperimmune ox sera prepared at the Muguga laboratory of the East African Veterinary

Research Organisation were also used.

Goat Sera: Eight Nigerian Dwarf goats were inoculated subcutaneously with the caprinized strain of rinderpest virus. Sera were obtained at different intervals after inoculation.

Pig Sera: Sera were obtained from 22 West African Dwarf pigs inoculated subcutaneously with the caprinized strain of rinderpest virus.

Rabbit Sera: Rabbits were first inoculated intravenously with rinderpest hyperimmune ox serum and then given multiple inoculations at different intervals intravenously and/or intraperitoneally with lapinized rinderpest virus. Sera were collected one to two weeks after the last inoculation.

TESTS

MEASLES HAEMAGGLUTINATION

Standard Technique: Measles haemagglutination (HA) was performed in WHO perspex plates[1] or in glass/plastic tubes (64 mm by 10 mm). Two-fold serial dilutions of the haemagglutinins were made in PBS (pH 7.2) in 0.2 ml volumes. To each dilution was added 0.1 ml of the diluent followed by 0.2 ml of an 0.5 per cent suspension of simian erythrocytes in Alsever's solution. The plates or tubes were held at 4, 22 or 37°C overnight, two hours and 60-90 minutes respectively. The highest dilution of haemagglutinins causing complete agglutination of the erythrocytes was taken as the end-point. The logarithm of the reciprocal of the end-point dilution was added to 0.7 to give the titre per ml. Tests were replicated.

Erythrocyte Suspensions: Blood samples from cats, cattle, dogs, fowls, geese, goats, horses, pigs, rabbits and sheep were obtained by venepuncture. Ferrets, frogs, guinea pigs, mice and rats were bled from the heart under anaesthesia. Human blood O, A and B types were obtained from the Haematology Department, Royal Infirmary, Edinburgh. Blood samples from rhesus, cynomolgus, patas (Cercopithecus aethiops) and talapoin (Cercopithecus talapoin) monkeys were obtained from the

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1. Prestware Ltd., London.

Department of Bacteriology, Manchester University and the Department of Anatomy, Birmingham University Medical School. However, most studies were conducted with baboon (Papio spp.) blood samples obtained from Arthur D. Little Research Institute, Musselburgh.

All specimens of blood were collected in Alsever's solution with sterile precautions. The samples were washed thrice with 5-10 volumes of sterile Alsever's solution and resuspended in equal volumes with the same solution.

All but the simian blood samples were used within 1-2 days after collection. Monkey blood was stored up to three months at $4^{\circ}C$. However, for routine haem-agglutination (HA) and haemagglutination-inhibition (HI) tests, blood preserved for not more than four weeks was used.

Effect of ions: The influence of monovalent or divalent ions on measles haemagglutination was tested by the methods outlined by Norrby (1962a). A sample of Behringwerke Tween-ether haemagglutinins was dialysed against deoinised distilled water. A 5 per cent glucose solution was used as the diluent. Baboon erythrocytes were washed five times in the diluent to which Nacl was added to a concentration of 0.0025 N; this concentration of the salt was necessary to obviate auto-agglutination. The effect of incorporation of sodium chloride, magnesium chloride and calcium lactate

in increasing concentrations on haemagglutination titres was then tested.

Effect on erythrocyte concentration:

Direct tests: Suspensions of baboon erythrocytes ranging from 0.10 to 12.00 per cent in Alsever's solution were tested in titrations of Behringwerke Tween-ether haemagglutinins, titrations with each cell concentration being replicated three times.

Adsorption tests: The capacity of a 2.5 per cent suspension of baboon erythrocytes to adsorb Tween-ether haemagglutinins was tested at different temperatures. A preparation of Behringwerke Tween-ether haemagglutinins having a titre of 2.6 $\log_{10}$ units per ml was distributed in 0.4 ml aliquots into small tubes. To these were added 0.4 ml of an 2.5 per cent of baboon erythrocytes in Alsever's solution. The mixtures were held at 4, 21, 37 or 45°C. Tubes were removed at different intervals, chilled and centrifuged at 3,000 r.p.m. for 5-10 minutes. The supernatant fluids were titrated for residual haemagglutinins.

In another trial the adsorption capacities of 2.5 and 5 per cent erythrocytes were compared by treating the suspensions with aliquots of Tween-ether haemagglutinins at 37°C for different intervals.

Effect of pH on haemagglutination: Preparations of Behringwerke Tween-ether and Wellcome heated haemagglutinins were diluted in equal volumes with buffer

solutions designed to give a pH range of 3 to 11.

Erythrocyte suspensions were made in the respective buffers to a final concentration of 0.5 per cent. The plates were held at room temperature for two hours and the titres were noted and compared.

In another study aliquots of a preparation of Tween-ether haemagglutinins having an activity of 64 HA units per ml were mixed in 0.5 ml volumes with 2.5 per cent baboon erythrocytes made in the respective buffers (pH 3 to 11). The mixtures were held in a 37°C water-bath for 30 minutes and then centrifuged at 3,000 r.p.m. for 20 minutes. The supernatant fluids were tested for residual haemagglutinins.

Adsorption studies were also carried out at different temperatures and range of pH with 2.5 per cent suspensions of erythrocytes from fowls and geese and the different mammalian species referred to before. Sixty-four units per ml of Tween-ether haemagglutinins were incubated with the suspensions for varying periods.

Treatment with neuraminidase: Stock solutions of neuraminidase[1] (Vibrio cholerae filtrate) were made in 0.15 M NaCl solution. Aliquots of a 10 per cent suspension of baboon erythrocytes in Alsever's solution in 4.5 ml volumes were mixed with 0.5 ml of appropriate dilutions of the enzyme to give final concentrations of

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1. The British Drug House Ltd., Poole.

0.03, 0.075, 0.15 and 0.30 mg/ml. Incubation proceeded for 30 minutes at 4, 22 or 37°C. The mixtures were chilled and centrifuged at 3,000 r.p.m. for 10-15 minutes and the supernatant fluids discarded. The erythrocyte pellets were resuspended in 25 ml of Alsever's solution and centrifuged at 3,000 r.p.m. for 10-15 minutes. The cells were reconstituted in Alsever's solution containing 0.1 per cent bovine serum albumin[1] to give a final concentration of 0.5 or 1.0 per cent. The sensitivities of untreated and neuraminidase-treated erythrocytes were compared using Behringwerke Tween-ether haemagglutinins. The tests were replicated.

In a second study, the sensitivities of neuraminidase-treated baboon, rhesus and cynomologus monkey erythrocyte suspensions were compared after treatment at 37°C for 30 minutes with 0.15 and 0.30 mg/ml of the enzyme.

Neuraminidase-treated (0.15 mg/ml) erythrocytes from cattle, fowls, geese, horses, human A, B and O groups, rabbits and sheep were tested to determine the reaction specificity of measles haemagglutination. Three samples of blood from each species and five of human O group were tested. The treatment was carried out at 37°C for 30 minutes.

Treatment with tannic acid: Aliquots of 0.1 ml of

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1. The British Drug House Ltd., Poole.

packed baboon erythrocytes were mixed with 0.19 ml of freshly prepared solution of tannic acid[1] in 0.15 M NaCl, the final concentrations of tannic acid ranged from 1:10,000 to 1:40,000. The mixtures were held at 4, 22 or 37°C for varying periods, from 5 to 60 minutes. Chilled veronal[2] buffer (pH 7.2) containing 0.1 per cent bovine serum albumin was added to 25 ml volume and the mixtures were centrifuged at 2,000 r.p.m. for 20 minutes. The cell pellet was reconstituted in 25 ml of the same diluent and packed at the same speed for 20 minutes. The cells were resuspended in 20 ml of Alsever's solution containing 0.1 per cent of bovine serum albumin. The sensitivities of untreated and tanned cells were compared using a preparation of Behringwerke Tween-ether haemagglutinins.

Treatment with formalin: The method of formolizing sheep erythrocytes described by Csizmas (1960) was followed with minor modifications.

In a sighting study packed erythrocytes from freshly collected baboon blood and blood stored for two days at 4°C were distributed into 0.5 ml aliquots, into glass beakers containing 50 ml of veronal buffer (pH 7.2). One to 10 per cent formalin[3] was dialysed at

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1. The British Drug House Ltd., Poole.
2. The Oxoid Division of Oxo Ltd., London.
3. Formalin Analar Grade, The British Drug House Ltd., Poole.

room temperature against the erythrocyte suspensions which were stirred for one hour. The cellophane tubes were punctured and the contents allowed to react with the erythrocytes for a further period of 30 minutes at room temperature. The suspensions settled at 4°C overnight. The supernatant fluids were discarded and the cells were suspended in 25 ml of chilled veronal buffer containing 0.1 per cent bovine serum albumin and centrifuged at 2,000 r.p.m. for 20 minutes. The sediments were resuspended in the same buffer and centrifuged again at 2,000 r.p.m. for 20 minutes. The processes were repeated 3-5 times and the packed cells were reconstituted in Alsever's solution containing 0.1 per cent bovine serum albumin. Titrations of Tween-ether haemagglutinins were carried out with formolized and unformolized erythrocytes. The tests were replicated.

In a second trial the effect of incubation temperature on the efficiency of formolization was investigated. Fresh baboon blood and baboon blood stored for four days were treated with three different concentrations of formalin, namely 0.5, 1.0 and 2.0 per cent, at 4, 22 and 37°C for two hours. The test was replicated three times.

In another study, baboon blood stored for different periods, from 2 to 14 days, were formolized at room temperature for two hours using formalin to give final

concentrations of 0.1 to 10 per cent.

Treatment with trypsin: Five per cent suspensions of baboon erythrocytes were dispensed into centrifuge tubes in 4.5 ml amounts. To the tubes were added trypsin solution in 0.15 M NaCl to give final concentrations of 10 and 100 mg/ml. The mixtures were held at 37°C for periods ranging from 10 to 50 minutes. Fifty units of soyabean trypsin inhibitor were added and the suspensions were chilled. The cells were sedimented by centrifuging at 2,000 r.p.m. for 20 minutes and reconstituted to 25 ml volume in Alsever's solution containing 0.1 per cent bovine serum albumin. The cells were packed again and resuspended to give 0.5 to 1 per cent concentrations in Alsever's solution. The effect of enzyme treatment was tested by titrating Tween-ether haemagglutinins using treated and untreated cells.

Treatment with oxidising agents: Five per cent baboon erythrocytes in Alsever's solution were incubated at 37°C for 30 minutes with two different molar concentrations of the oxidising agents, potassium permanganate, potassium periodate, hydrogen peroxide and iodine. The mixtures were diluted to ten times their volume with chilled veronal buffer (pH 7.2) containing 0.1 per cent bovine serum albumin. Cells were sedimented and washed with the buffer solution 2-3 times as before. Treated and untreated cells were compared.

Baboon Erythrocyte Membranes:

Preparation: About 50 ml of packed baboon erythrocytes were lysed with 2 litres of deoinised distilled water. The suspension was allowed to stand overnight at 4°C and two-thirds of the supernatant fluid were decanted. To the remainder were added about 500 ml of 0.15 M NaCl solution and the mixture was stirred and centrifuged in 250 ml amounts in an ultracentrifuge[1] at 10,000 r.p.m. for one hour. The pellet was resuspended in a litre of 0.15 M NaCl solution and centrifuged again at the same speed for one hour; the process was repeated five times.

The pellet was reconstituted in 25 ml of 0.15 M NaCl solution and disintegrated in a sonic disintegrator at 10 Kc for five minutes. The suspension was dialysed against 0.15 M NaCl for 48 hours at 4°C and concentrated against "carbowax". The final preparation was distributed into 0.5 ml aliquots and stored at -75°C.

Adsorption tests: Serial two-fold dilutions of the erythrocyte membrane suspension were made in triplicate in glass tubes in 0.5 ml amounts. To the dilutions were added 4 units of Behringwerke Tween-ether haemagglutinins in 0.5 ml amounts. The tubes were held at 37°C for one hour and then centrifuged at 3,000 r.p.m. for 20 minutes. The supernatant fluids were tested for

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1. "MSE ultracentrifuge 18", Measuring and Scientific Equipment Ltd., London.

haemagglutinins by adding 0.5 ml of an 0.5 per cent suspension of baboon erythrocytes and incubating the mixtures at 37°C for one hour. The highest dilution of the membrane suspension that showed haemagglutination was deemed to contain 1 adsorption unit of the preparation. The logarithm of the reciprocal of the end-point dilution was added to 0.7 to give the titre per ml.

In a second study 0.5 ml aliquots of six different batches of Behringwerke Tween-ether haemagglutinins were mixed in centrifuge tubes with 0.5 ml of baboon erythrocyte membrane suspension having an adsorption titre of 2.5 $\log_{10}$ units per ml. The mixtures were incubated at 37°C for one hour, chilled on ice and centrifuged at 5,000 r.p.m. for 20 minutes at 4°C. The supernatant fluids were tested for residual haemagglutinins.

In a third study, the effect of the temperature of incubation on the rates of adsorption of Behringwerke Tween-ether haemagglutinins by baboon red cell membranes was investigated. A preparation of the membranes having an adsorption titre of 2.8 $\log_{10}$ units per ml was dispensed into centrifuge tubes in 0.40 ml amounts. The tubes were divided into two lots. Behringwerke Tween-ether haemagglutinins having a titre of 2.9 $\log_{10}$ units per ml were added in 0.40 ml amounts. The tubes were incubated respectively at 22 and 37°C. Similarly, a second preparation of the erythrocyte membranes having a titre of 3.1 $\log_{10}$ units per ml were dispensed into

two sets of tubes. Tween-ether haemagglutinins were added and the mixtures were incubated respectively at 30 and 37°C.

The tubes were removed at intervals varying from 10 to 80 minutes, chilled and centrifuged at 5,000 r.p.m. for 20 minutes. The supernatant fluids were titrated for haemagglutinins, the titrations being replicated.

Formalin-treatment of baboon erythrocyte membranes: A preparation of baboon erythrocyte membranes having an adsorption titre of 2.8 $\log_{10}$ units per ml was distributed in aliquots of 0.9 ml in glass tubes. Formalin solutions in 0.15 M NaCl were added in 0.1 ml amounts to give final concentrations ranging from 1 to 10 per cent. Three to four tubes were used for each concentration of formalin. The tubes were held in a 37°C water-bath for 30 minutes and then chilled on ice. The contents were transferred into Visking cellophane tubes and dialysed for 24 hours against 0.15 M NaCl solution at 4°C.

The contents of the Visking tubes were transferred to centrifuge tubes in 0.5 ml amounts and 0.5 ml of Behringwerke Tween-ether haemagglutinins having a titre of 3.7 $\log_{10}$ units per ml were added. The mixtures were incubated at 37°C for one hour and then spun at 5,000 r.p.m. for 20 minutes at 4°C. The supernatant fluids were titrated for haemagglutinins.

The effect of treatment with different concentrations of formalin on the adsorption titres of baboon

erythrocyte membranes was compared with that of treatment of 1 per cent baboon erythrocyte suspensions on their capacity to adsorb the haemagglutinins.

Thermal inactivation of baboon erythrocyte membranes: The effect of heat on the adsorption titres of baboon erythrocyte membranes was tested by exposure of aliquots having an adsorption titre of 2.5 $\log_{10}$ units per ml to 50 or 55°C for intervals ranging from 10 to 120 minutes and titrating the heated suspensions for adsorption activities.

ELUTION

Packed baboon erythrocytes were incubated for 24 to 48 hours at 4°C with Behringwerke Tween-ether haemagglutinins. The mixture was centrifuged at 3,000 r.p.m. for 20 minutes and the supernatant fluid was tested for residual haemagglutinins. The sedimented cells were incubated again with the haemagglutinins at 4°C until no further adsorption occurred. The cells were then suspended in 0.15 M NaCl and in buffer solutions in the range of pH 4 to 10. Aliquots were held at 4, 22 and 37°C for seven days. Suspensions were centrifuged and the supernatant fluids tested for haemagglutinins.

In another study, the cells with adsorbed haemagglutinins were treated with 0.15 mg/ml of neuraminidase at room temperature for intervals ranging from 30

minutes to four hours. The suspensions were then centrifuged and the supernatant fluids tested for haemagglutinins.

In a third study neuraminidase-treated (0.15 to 0.5 mg/ml) baboon erythrocytes were adsorbed with Tween-ether haemagglutinins at 4°C for 24 hours. The suspensions were centrifuged and the cells were reconstituted in buffer solutions in the pH range of 4 to 10. The mixtures were held at 4°C for two weeks and tested at intervals of two days for evidence of elution of the haemagglutinins.

HAEMADSORPTION

Standard Technique: At the height of the CPE, monolayers of measles-infected cell cultures were rinsed 2-3 times with PBS or Hank's BSS. A 3 per cent suspension of monkey erythrocytes in PBS or Hank's BSS was added in 0.2 ml amounts. The cultures were incubated at room temperature or 37°C for 45 and 30 minutes respectively. The unadsorbed cells were decanted and the monolayers were rinsed 2-3 times with PBS or Hank's BSS. Cultures were examined microscopically for foci of haemadsorption (Fig. 5).

Reaction Specificity: Freshly made 3 per cent suspensions of erythrocytes from 3 avian and 11 mammalian species were tested for haemadsorption on measles-infected monolayers. Replicate cultures were

used for each cell suspension. Cultures treated with baboon erythrocytes constituted the positive controls. Uninfected cell cultures treated with baboon erythrocytes served as the negative controls.

In a second study, suspensions of rhesus, cynomologus or baboon erythrocytes were incubated with human influenza A haemagglutinins, Newcastle Disease Virus inactivated haemagglutinins or stock pools of canine distemper CTVM, the Rockborn and Onderstepoort virus strains at 4°C overnight. The suspensions were washed three times in Hank's BSS, resuspended in Hank's BSS to 3 per cent concentration, and tested on measles-infected monolayers for haemadsorption.

Effect of pH: Haemadsorption tests were carried out with erythrocytes of different mammalian and avian species suspended in buffer solutions of the pH range 5-10 at 4, 22 and 37°C for 120, 45 and 30 minutes respectively. As positive controls, baboon erythrocytes were tested for haemadsorption under identical conditions of temperature and pH. Four samples of frog erythrocytes were also tested on measles-infected monolayers.

Other Studies: The relationships between the onsets of the CPE and haemadsorption were studied with reference to the age of the cell culture, the type of the cells and also the dose of the infective virus.

The influence of treatment of baboon erythrocytes

and erythrocytes from different species of mammals and birds with neuraminidase and of the treatment of baboon erythrocytes with formalin and tannic acid on measles haemadsorption was examined. Likewise, the effect of pre-treatment of measles-infected and uninfected monolayers with neuraminidase on the intensity of haemadsorption of baboon erythrocytes was also investigated.

HAEMAGGLUTINATION-INHIBITION

Standard Technique: Sera were diluted 1:2 in PBS and inactivated at 56°C for 30 minutes. To adsorb possible non-specific inhibitors of measles haemagglutination, a 25 per cent suspension of acid-washed kaolin in borate buffer (pH 8.0) was added in equal volumes and the mixtures were held for 20 minutes at room temperature. The suspensions were centrifuged at 2,500 r.p.m. for 20 minutes and the supernatant fluids were treated with packed or a 50 per cent suspension of simian erythrocytes in PBS in the ratio of 4:1. The mixtures were incubated at room temperature for 2-3 hours or overnight at 4°C and then centrifuged at 2,000 r.p.m. for 20 minutes. The sera were deemed to have been diluted 1:5.

Serial 2-fold dilutions of the sera in PBS (pH 7.2) were made in WHO plates in 0.2 ml amounts. Four units of Behringwerke Tween-ether haemagglutinins were added in 0.2 ml amounts and the plates were held at room temperature or 4°C for two hours and overnight

respectively. An 0.5 per cent suspension of simian erythrocytes in Alsever's solution was added in 0.1 ml volumes. The plates were incubated at 37°C or room temperature for 1-2 hours. The highest dilution of the serum that gave complete inhibition of measles haemagglutination was taken as the end-point. The $\log_{10}$ of the reciprocal of the end-point dilution was added to 0.7 to express the titres per ml.

The suspension of haemagglutinins used was titrated in triplicate in 0.2 ml amounts in 2-fold series to give 4, 2, 1 and $\frac{1}{2}$ units. To the dilutions were added 0.1 ml of the diluent and 0.2 ml of the simian erythrocyte suspension.

Serum controls consisted of 0.2 ml of the first dilution of the test sera, 0.2 ml of the diluent and 0.1 ml of erythrocytes. Erythrocyte controls contained 0.4 ml of the diluent and 0.1 ml of the erythrocyte suspension.

Reaction Specificity: Measles HI tests were carried out with reference sera containing neutralising antibodies to human influenza types A, B and C, parainfluenza types 1, 2, 3 (human and bovine) and 4, respiratory syncytial virus, mumps, fowl plague and Newcastle Disease.

Non-specific Inhibition: Sera from fowls and 11 species of mammals were examined for non-specific inhibitors of measles HA.

A sighting trial determined the comparative

efficiencies of pre-treatment of a few specimens of animal sera with kaolin, acetone and the heparin-manganous chloride mixture on the removal of non-specific inhibitors. The treatments of sera with kaolin, acetone and the heparin-$McCl_2$ mixture were on lines similar to those respectively described by Spence (1960), Kunita et al. (1963) and Mann, Rossen, Lehrich and Kasel (1967).

Two trials were conducted to study the effect of kaolin on specific HI antibodies in anti-measles sera. Specimens of sera diluted 1:2 in PBS were heat-inactivated and then incubated in equal volumes with a 25 per cent solution of kaolin in borate buffer (pH 8.00) at 4, 22 or 37°C for 24 hours. Sera were clarified by centrifugation and tested for HI titres. The titres were compared with the titres obtained with untreated sera. In the second trial, the effect of prolonged incubation with kaolin solution at 4°C on the specific HI antibody titres of the sera was investigated.

Non-specific Agglutinins: Avian and mammalian sera were tested for titres of non-specific agglutinins for simian erythrocytes. Serial double-fold dilutions of the sera in PBS were incubated with equal volumes of a 1 per cent suspension of monkey erythrocytes and incubated at room temperature for 60 minutes.

The effect of treatment with different concentrations of monkey erythrocytes on the adsorption of the heterologous haemagglutinins in animal sera was also

examined.

In another study the usefulness of monkey erythrocyte membrane suspensions for the adsorption of the natural agglutinins for monkey erythrocytes in animal sera was investigated.

Other Studies: The effects of antigen concentration, temperature and periods of incubation on the HI titres were studied with specimens of anti-measles sera from different sources. All tests were replicated.

The influence of the type of antigen on HI titres was studied in some detail using crude preparations of the CTVM haemagglutinins, Wellcome heat-inactivated haemagglutinins, Behringwerke Tween-ether and CTVM deoxycholate-treated haemagglutinins against anti-measles, anti-distemper and anti-rinderpest sera. The efficiencies of the antigens were compared.

A study of the thermostability of measles HI antibodies was carried out by exposing anti-measles sera of different sources to 70°C for different periods and then titrating the treated sera for HI antibodies.

HAEMADSORPTION-INHIBITION

Standard Technique: Sera were pre-treated on the lines similar to those described for measles HI test. Measles-infected monolayers at the height of the CPE were tested for haemadsorption. Cultures showing a similar degree of the CPE were then selected. The

monolayers were washed 2-3 times with Hank's BSS or PBS and dilutions of the test serum in 2-fold series were added in 0.1 ml amounts to the cultures, using four tubes for each dilution. The tubes were held at room temperature for two hours or at 37°C for one hour. The sera were decanted and the monolayers rinsed 2-3 times with Hank's BSS or PBS. A 3 per cent suspension of baboon erythrocytes in Hank's BSS was added in 0.1 ml amounts and the cultures were incubated at room temperature or 37°C for 45 and 30 minutes respectively. The monolayers were washed a few times with Hank's BSS or PBS and then examined microscopically for haem-adsorption. The highest dilution of the serum giving complete inhibition of haemadsorption was taken as the end-point. Fifty per cent end-points were calculated according to Reed and Muench (1938) method. Titres were expressed in $\log_{10}$ units per ml.

A positive serum diluted to contain HAd.1 units and replicate infected cultures seeded with a 3 per cent suspension of baboon erythrocytes served as the positive serum and haemadsorption controls in the test.

COMPLEMENT-FIXATION TEST

Standard Technique: The technique described by the WHO Expert Committee on Respiratory Virus Diseases (1959) was followed. Tests were carried out in WHO plates.

Sera: Sera were inactivated at 56°C for 30 minutes

after diluting 1:4 in veronal-NaCl buffer.

Diluent: Veronal-NaCl buffer containing 0.1 per cent bovine serum albumin was used.

Haemolytic system: Sheep blood was collected in Alsever's solution and washed three times in 0.15 NaCl and preserved in Alsever's solution for periods of up to four weeks. A 3 per cent suspension of the erythrocytes in the diluent was sensitized with an equal volume of a dilution of horse or rabbit haemolysin[1] containing five minimum haemolytic doses (MHD). The mixture was held at 37°C for 10 minutes or at room temperature for 30 minutes.

Complement: Freeze-dried guinea pig complement was obtained from Burroughs Wellcome Ltd., and was reconstituted according to their instructions.

Antigens: Cell culture antigens were used in the measles CF tests. For canine distemper tests cell culture and tissue antigens were used.

To avoid non-specific fixation of complement, measles CF antigens prepared in FL cells were used to test antibodies in the sera of rabbits, guinea pigs and dogs inoculated with the virus or haemagglutinins obtained from simian cell lines. Likewise, sera of rabbits inoculated with distemper virus grown in MDCK cells were tested for CF antibodies using CF antigens

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1. Burroughs Wellcome & Co., London.

obtained from fowl embryo fibroblast cultures infected with the Onderstepoort strain of canine distemper virus. Dog anti-distemper sera were tested with MDCK cell-culture antigens or distemper-infected canine tissue antigens.

Titration of antigens: Antigen titrations were carried out by standard methods using known positive and negative sera (Cruickshank, 1965). Commercially prepared measles[1,2] and canine distemper[2] CF antigens were used initially to standardize the prepared antigens. The latter then served as reference antigens in the titrations of subsequent batches of measles and canine distemper CF antigens.

Complement titration: Titration of complement was done according to the manufacturer's instructions.

The test: All reagents were pre-chilled at 4°C. Serial two-fold dilutions of sera in the diluent were carried out in 0.1 ml volumes in duplicate. The dilutions ranged from 1:8 to 1:256. Four units of specific CF antigens were added to one series of dilutions in 0.1 ml volumes. The second series of dilutions received similar amounts of the negative control antigens. A dilution of complement containing two full haemolytic units in 0.1 ml was then added.

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1. Wellcome Research Laboratories, Beckenham.
2. Winthrop Biologicals Ltd., Newcastle-on-Tyne.

Specimens of a known positive and negative sera were used as control sera. Antigen controls consisted of 4 units of specific antigens and a standardized dilution of the negative control antigens in 0.1 ml amounts to which were added 0.1 ml of the diluent and 0.1 ml of the complement. Controls of the sera under examination comprised the first two dilutions of the sera, namely 1:8 and 1:16 in 0.1 ml amounts, 0.1 ml of the diluent and 0.1 ml of complement. The amount of complement used in the test was titrated in triplicate to give 2, 1, $\frac{1}{2}$ and $\frac{1}{4}$ units in 0.1 ml volumes. Diluent was then added in 0.2 ml amounts.

The plates were held in the refrigerator for 20-24 hours and then incubated at 37°C for 20-25 minutes. Freshly prepared suspensions of 3 per cent sheep erythrocytes optimally sensitized with the rabbit haemolysin were added in 0.1 ml amounts. Sensitized and unsensitized cells dispensed in 0.1 ml amounts and mixed with 0.3 ml of the diluent constituted the cell controls.

Plates were then incubated at 37°C for 45 minutes. The contents were shaken 2-3 times every 15 minutes. Final readings were taken after further incubation in the refrigerator for 2-4 hours. The highest dilution of the serum showing 50 per cent haemolysis was taken as the end-point. Titres were expressed as $\log_{10}$ units per ml. All tests were replicated.

When sera reacted both with specific and negative control antigens the complement dilution technique was employed using 2, 4, 6 and 8 full units of the complement. A similar procedure was followed in determining the specificity of antigens obtained from distemper-infected and uninfected tissues.

The modified direct CF test described by Boulanger (1960) was used to demonstrate CF antibodies to distemper CF antigens in the sera of cattle.

Parameters of the CF Test: The effects of incubation temperature, antigen concentration and the number of units of complement used on measles and distemper CF antibody titres in the sera of the respective natural and experimental hosts of the two viruses were investigated and the results compared.

IMMUNO-DIFFUSION TEST

Standard Technique: Ionagar No. 2[1] was dissolved in an autoclave at 121°C to give a 1 per cent solution in deionised distilled water. Thiomerasal[2] solution was added to give a 0.04 concentration. The agar was poured into plastic petri dishes in 4.5 ml amounts to form a layer 2 mm thick. Circular wells, 5mm in diameter were cut in the solidified agar with a standard

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1. The Oxoid Division of Oxo Ltd., London.
2. The British Drug House Ltd., Poole.

punch giving an hexagonal pattern. The diffusion distance between wells was 2.5 mm.

Most distemper immuno-diffusion tests were carried out in larger petri dishes containing solidified agar. The wells were cut with a Feinberg Agar Gel Cutter[1] with a larger central well (12.5 mm diameter) and six small peripheral wells (4 mm diameter). The distance between the central and peripheral wells was 6 mm.

Measles: Concentrated cell culture fluids and Behringwerke Tween-ether haemagglutinins were used as antigens to demonstrate ID antibodies in anti-measles sera. For titration of the antigens the peripheral wells were charged with 0.1 ml of serial 2-fold dilutions of the antigen in a clock-wise fashion. The reference serum was placed in the central well. Tests were always carried out in replicate plates. The plates were incubated in a humid atmosphere at room temperature for periods of up to seven days. The highest dilution of the antigen giving a clear-cut precipitation line with the antiserum was considered as the end-point. Titres were expressed as $\log_{10}$ units per ml.

Titration of anti-measles sera were carried out on similar lines. The central well was charged with the reference ID antigens.

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1. Shandon Scientific Company Ltd., London.

Canine distemper: Suspensions of infected tissues and cell culture antigens were used. For the detection of CF antigen activity in infected tissues from alleged clinical cases of distemper, a piece of the tissue was minced with scissors and the mince was transferred to the central well. The specificity of any unknown reaction was checked by the merging of the precipitation line with that between wells containing reference serum and reference antigen.

Reaction Specificity: Aliquots of anti-measles and anti-distemper sera were incubated at 4°C for 24 hours with the homologous cell culture or tissue ID antigen suspensions and also with the concentrated uninfected cell cultures or tissue suspensions. The mixtures were spun at 3,000 r.p.m. for 20 minutes and titrated. The titres were compared.

Studies of Parameters: The effects of incubation temperature, antigen concentration and antibody concentration on the rates of appearance of the precipitation lines were studied using replicate plates.

Other Studies: Tests were carried out to determine the effect of exposure to 60°C for 30 minutes on the ID titres of Behringwerke Tween-ether haemagglutinins and the concentrated cell culture fluids. The effect of heat on the number of precipitation lines induced by these antigens when diffused against anti-measles serum was also investigated. Likewise, the effect of heat on

the titres of the distemper ID tissue antigens was studied.

In another trial the influence of sodium chloride concentration on measles precipitation reactions was studied by incorporating salt solution of different molarities in the gel.

The serological cross-relationships between measles, rinderpest and distemper were examined in direct tests and adsorption tests using measles and distemper antigens and measles, rinderpest and distemper hyperimmune sera of various sources.

NEUTRALISATION TEST

Virus Pools: Since the fluids from measles and canine distemper-infected cell cultures harvested at the height of the CPE were likely to contain virus particles inactivated by the temperature of incubation, the method described by Drevo and Mares (1967) to obtain harvests comprising predominantly of live virus particles was followed.

The Molly Hill strain of measles virus and the Rockborn strain of canine distemper virus were inoculated into three to four days' old FL and MDCK cells respectively using undiluted inocula. The Onderstepoort strain of canine distemper virus was seeded into one day-old primary fowl embryo fibroblast cultures. At the time when 50 per cent cells in the cellsheets manifested cytopathic changes, the fluids were removed

and the monolayers washed 2-3 times with Hank's BSS. A maintenance medium consisting of equal volumes of bovine amniotic fluid and medium 199 to which foetal bovine serum to a final concentration of 5 per cent was prepared afresh and dispensed into the culture flasks. The cultures were incubated for a further period of four hours at 37°C. The fluids were harvested, clarified by centrifugation at 3,000 r.p.m. for 15 minutes and then the supernatant fluids were dispensed into 2 ml aliquots for storage at -75°C.

Thermal Inactivation Studies: The thermal decay of measles and distemper viruses was studied by exposing aliquots of the viruses to 4, 22 or 37°C for different intervals and titrating the treated preparations. Eight to ten tubes were used for each dilution. The results of the studies formed the basis of the related investigations of the influence of the dose of the virus on the titres of neutralizing antibodies to measles and distemper in the different specimens of sera tested.

Standard Technique: Sera were not inactivated. They were clarified by centrifugation in a Hemings filter.[1] An initial 1:10 dilution was made in maintenance medium consisting of medium 199 buffered with sodium bicarbonate solution and supplemented with 5 per cent foetal calf serum. Thereafter serial two-fold dilutions were

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1. Carlson-Ford Sales Ltd., Ashton-under-Lyne.

made in the same medium in 1 ml amounts. Measles titrations were carried out in 3-4 days' old FL or AV_3 cells and occasionally in LLC-MK2 cells. For distemper titrations MDCK cells were used.

The monolayers were rinsed thrice with Hank's BSS and seeded with the different dilutions of the serum previously incubated with equal volumes of 200 TCD_{50} of virus per 0.1 ml at room temperature for two hours. Four to five tubes were used for each serum dilution and the tubes were seeded with 0.2 ml of the serum-virus mixture. Adsorption proceeded for two hours at room temperature or for one hour at 37°C. The monolayers were then washed three times with Hank's BSS and maintenance medium was added. The cultures were incubated at 37°C.

Simultaneously the TCD_{50} dose of the virus used in the test was incubated for two hours at room temperature and then diluted in the maintenance medium in ten-fold series. The undiluted inoculum and the first two dilutions were then seeded into the cultures using four tubes per dilution.

Measles cultures were examined for evidence of haemadsorption 12-14 days after inoculation using a 3 per cent baboon red cell suspension. Fifty per cent end-points of the inhibition of haemadsorption were calculated by the method of Reed and Muench (1938).

Distemper cultures were inspected for the CPE after

14 days; visual assessment was confirmed by microscopic examination of cover-slip cultures. The neutralising antibody titres were expressed as $\log_{10}$ units per ml after calculating the 50 per cent end-points of inhibition of the CPE.

ANALYSES

Analyses of data were carried out by standard statistical techniques (Snedecor, 1957). In the evaluation of the test parameters and the relationships between variables in other studies, regression coefficients of the lines of the best fit were calculated (Snedecor, 1957). Comparisons of regressions were based on the method described by Dawkins (1968).

Half-life values were estimated according to the formula:

$$t\ \frac{1}{2} = \frac{0.3}{b}$$

where t is the exposure temperature and b is the regression coefficient. Arrhenius plots were calculated by the method described by Pollard (1953) and tests for significance were carried out by analysis of variance (Snedecor, 1957).

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PRODUCTION OF VIRUSES AND ANTIGENS

BEHAVIOUR OF MEASLES VIRUS IN CELL CULTURES

Cytopathogenicity in Primary Cell Cultures: The virulent Belfast strain of measles virus induced cytopathogenic changes only in primary cultures of PMK cells (Table 3). On first isolation the strain caused cytopathogenic effects after five days incubation at 36°C. After passage the strain induced cytopathogenic effects in four days.

The Holly Hill, Colindale and Beckenham strains differed slightly in behaviour after serial propagation in PMK and PCK cells and fowl embryo fibroblasts (Tables 4-6). Whereas the Beckenham strain was readily adapted to fowl embryo fibroblasts it needed four blind passages before evidence of replication of the other two strains of the virus was manifest. Even so, growth of the Holly Hill and Colindale strains in fowl embryo fibroblasts was poor and was not accompanied by any recognizable pattern of CPE.

Both in PCK and PMK cells serial passage entailed a concomitant diminution in the time of induction of the CPE. At the fifth passage level, the CPE set in regularly between the fourth and fifth day in PMK cells and the seventh to ninth day in PCK cells. The CPE in the PMK cell cultures was characterized not only by the rapidity in onset but also by the quick multicentric spread; disintegration of the cell sheet was generally

complete by the twelfth to fourteenth day with lower dilutions of inocula. In the PCK cells, on the other hand, infection tended to remain focal in distribution. Even with neat inocula only about 50 per cent of the cells appeared to be infected and complete degeneration of the cell sheet was not observed.

The virus could not be adapted to primary rabbit, guinea pig, mouse, rat, calf, lamb, hamster and cat kidney cells, and to bovine foetal lung fibroblasts. Likewise, the virus failed to infect leucocyte cultures from healthy rabbits, calves and sheep.

Cytopathegenicity in Cell Lines: At the fifth passage level in PMK cells, the virulent strain of measles virus was successfully adapted to FL, Hep-2 and LLC-MK2 cells but not to MDCK cells (Table 7). Pre-treatment of MDCK cells with actinomycin-D had no ameliorative effect on the apparent refractoriness of the cells to infectivity with the virulent virus.

The adapted strains replicated with ease in the human and simian established cell cultures (Tables 8-10). The Holly Hill and the Beckenham strains were adapted to MDCK cells but the onset of the CPE was protracted, the spread of infection poor and the involvement of the cell sheet was focal and self-limiting.

Effect of age of cells: The influence of the age of the cells on the time of onset of the CPE in FL cultures was studied using the Holly Hill strain of the

virus. Fully-formed monolayers were dispersed with sodium versenate-trypsin mixture and approximately 6-8 x 10^6 cells/ml were seeded into tubes with cover-slips and allowed to monolayer at 36°C. Simultaneously, an aliquot of the cell suspension was centrifuged in outgrowth medium twice at 1,000 r.p.m. for five minutes and the cells were reconstituted to a density of 6-8 x 10^6 cells/ml in outgrowth medium containing $10^{4.8}$ TCD_{50}/ml of the virus. The tubes were held at 36°C in a stationary position for four hours. The outgrowth medium was then replenished. Subsequently, batches of five tubes were inoculated with the virus at days, 1, 2, 3, 4, 6, 8 and 10, and incubated at 36°C after an adsorption period of two hours followed by renewal of outgrowth medium. Visual assessment of the CPE in the inoculated tubes were confirmed by examination of strained preparations of cover-slip cultures. The relationship between the age of the cells at the time of infection with the virus and the time of induction of the CPE was linear and significant (Fig. 6).

Infectivity Titres: Differences in viral titres in different cell cultures were significant (F = 17.68**, d.f. 6, 17) (Table 11). The cell types fell into three significant groups and within each group a gradient existed (Table 12).

Irrespective of the type of cell culture, the mean titres induced by the adapted strains were higher than

those induced by the virulent strain. The virulent strain, moreover, grew poorly in PCK cells and failed to grow and induce CPE in FEF cells.

Irrespective of the strain of virus, the highest titres occurred in cultures of FL cells.

Cytomorphology:

Virulent strain: On its isolation in PMK cells the virulent Belfast strain of measles virus induced the formation of syncytia and stellate cells after five days. The number of syncytia was few and their size small. They contained up to eight nuclei which were arranged like a string of pearls. Approximately one quarter of the syncytia carried cytoplasmic inclusion bodies; there were also a few nuclear inclusions. Stellate cells were also recognized, and they generally occurred in the vicinity of foci of desquamation in the cell sheet.

After the third passage the CPE appeared consistently earlier. With undiluted inocula syncytia were detected in stained preparations on the second day post-inoculation of three days' old PMK cells. Their number and size progressively increased in the next three to four days. On the seventh day several foci in the cell sheet were studded with numerous small syncytia containing 20-30 nuclei. Usually the nuclei were distributed as compact clusters in the centre, the peripheral expanse of cytoplasm showing granularity and

moderate to marked acidophilia. Small to medium-sized multiple vacuoles were also present in the fringe of the cytoplasm. Nearly half the syncytia had multiple cytoplasmic inclusion bodies but less than five per cent of the syncytia had nuclear inclusions. Cytoplasmic inclusions were also easily recognized in uninucleate cells but nuclear inclusions were hard to demonstrate. On the ninth or tenth day the syncytia had considerably enlarged and some of these carried up to 100 nuclei. Vacuolization of the cytoplasm was a prominent and constant feature and in some syncytia often tended to obscure the cytological features. Most syncytia still contained cytoplasmic inclusions but not nuclear inclusions. In cultures seeded with higher dilutions of the virus syncytia were present up to the sixteenth day post-inoculation. Examination of the deposit from centrifuged tissue culture fluids revealed increasing numbers of clumps of degenerate syncytia from the eighth day onwards (Fig. 7).

Adapted strains: Among the adapted strains, the Holly Hill strain was studied in a greater variety of cell systems than the Colindale and the Beckenham strains. There was little difference in the cytopathology of the infection induced by the Holly Hill and the Colindale strains in PCK and PMK cells (Figs. 8 and 9). On the other hand, in their first passage in FL cells only the Holly Hill strain produced a few

nuclear inclusions, and the cultures infected with the Colindale strain contained only the cytoplasmic inclusions. From the second passage onwards, FL and Hep-2 cells infected with the Colindale strain showed much fewer cytoplasmic inclusions which were also very much reduced in size. At the fifth passage level, the inclusions were no larger than granules but had a distinct halo. Passage of the Holly Hill strain in FL and Hep-2 cells did not entail any change affecting the numer and size of the cytoplasmic inclusions but nuclear inclusions were not detected after the second passage.

In LLC-MK2 cells infected by the three adapted strains cytoplasmic inclusions were hard to demonstrate (Fig. 10). Likewise, the CPE of the Holly Hill strain in Bsc-1 and Vero cells and of the Beckenham strain in Hep-2 cells was characterized predominantly by the occurence of large syncytia without evidence of the presence of cytoplasmic inclusions.

The effect of interpolation in PMK cells on the nature and magnitude of the CPE was investigated with the Beckenham strain in its fifth serial passage in LLC-MK2 cells. Cytoplasmic inclusions appeared in the first PMK back passage and increased in numbers and size through the third serial passage. A few nuclear inclusions were also recognized. Both cytoplasmic and nuclear inclusions were well-defined in the fourth and fifth passages but their numbers were not enhanced.

There was evidence of replication of the Holly Hill and Colindale strains in primary fowl embryo fibroblasts when assay of infectivity was carried out in FL cells, but the CPE did not follow any recognizable pattern; virus titres were poor (Table 13). The Beckenham strain, however, produced a CPE that was characterized by the presence of a few, small syncytia comprising of four to five hyperchromatic nuclei. There was also a few enlarged cells with occasional long stringy cytoplasmic processes. Vacuolization of the cytoplasm was an additional feature. The changes were reproducible when undiluted inocula were used to infect day old fibroblasts and appeared by the fifth or sixth day post-inoculation. On the other hand, non-specific degenerative changes characterized by the presence of rounded off cells with pyknotic or karyorrhectic nuclei, stellate and fusiform cells set in a day or two later. Acridine orange staining revealed no demonstrable inclusion bodies in the infected fibroblasts.

Dose effect: The influence of the dose of the virus on the onset of the CPE was studied by infecting three days' old FL cells with the virulent Belfast and the Holly Hill strains of measles virus. An inverse, linear and significant relationship was detected between the dose of virus used and the first appearance of the cytological hallmarks such that higher doses induced earlier CPE ($F = 100.76^{**}$ and $F = 91.37^{**}$ respectively

d.f. 1, 116) (Table 14). Similarly, the onset of the nuclear inclusion bodies in the cells infected with the Belfast strain of measles virus was inversely and significantly related to the dose of virus (F = 79.61**, d.f. 1, 38). Nuclear inclusions appeared later than the syncytia and cytoplasmic inclusions. Comparison of the regressions of the onset of syncytia and cytoplasmic inclusions on the dose in respect of the Holly Hill strain of measles virus revealed significant differences both in the slopes and in the levels (F = 7.25** and F = 5.33* respectively, d.f. 1. 116). On the other hand, with the Belfast strain, the two regressions had similar slopes (F = 2.03, d.f. 1, 116) but the levels differed significantly (F = 22.87**, d.f. 1, 116). Moreover, comparison of the combined regression of the onsets of the syncytia and cytoplasmic inclusions on the dose with the regression of the onset of the nuclear inclusions induced by the Belfast strain showed significant differences in the slopes and levels (F = 10.25** and F = 48.16** respectively, d.f. 2, 154) (Table 15).

Incubation temperature effect: The incubation temperature did not influence the onset and severity of the CPE significantly (F = 0.03, d.f. 1, 15). The median values of the day of onset of syncytial formation in cultures held at 33°C, 36°C and 39°C were 3.6, 3.3 and 2.8 days respectively. On the other hand, nuclear

and cytoplasmic inclusions were relatively fewer and smaller in size in cultures incubated at 33°C than in cultures held at higher temperatures.

Host cell effect: The effect of the host cell type on measles CPE was profound. In all the mammalian cell cultures virus replication was invariably associated with the development of polykaryocytes and cytoplasmic inclusions. Nuclear inclusions were, however, recognized only in PMK and FL cells (Table 16). Even in these cultures, nuclear inclusions were difficult to demonstrate three to four days after the onset of CPE.

In both frequency of occurrence and in their morphology syncytia varied in the difficult mammalian cell cultures studied. Whereas the syncytia were, as a rule, large and numerous in infected PMK cells, they were greatly reduced in size and number in PCK and primary ferret kidney cells (PFK). The cytoplasmic inclusion bodies in the two latter cultures were also smaller and poorly defined. Some batches of uninfected ferret and canine kidney cells contained large cytoplasmic granules which were difficult to differentiate from the measles-specific cytoplasmic inclusions in uninucleate cells.

Replication of the virulent Belfast strain in FL, AV_3, Hep-2 and Bsc-1 cells was associated with the production of large syncytia and fairly numerous cytoplasmic inclusion bodies but the syncytia were smaller

and relatively fewer in number in WI-38, HeLa and Vero cells. Cytoplasmic inclusion bodies were also not readily demonstrable in these cells.

Serial passage of the virulent Belfast strain of measles virus in FL and LLC-MK2 cells resulted in the reduction in size and number of the inclusion bodies. Nuclear inclusions were not detected in FL cells infected with the third passage virus even when undiluted inocula were used. Likewise, in FL and LLC-MK-2 cells the fourth passage virulent virus produced small, multiple granular cytoplasmic inclusions. In acridine orange stained cultures these bodies fluoresced a brilliant green but they were difficult to recognize in haematoxylin and eosin stained cultures.

MEASLES HAEMADSORPTION

The adhesion of monkey erythrocytes to the surfaces of measles-infected cells was pathognomonic. The union was irreversible and was prevented by pre-incubation of the infected cell sheets with anti-measles serum.

Relation to Infective Virus: Development of changes in the infected cells demonstrable by haemadsorption parallelled the development of infective virus irrespective of the virus strain or type of cell culture. In general, haemadsorption was recognized only in cultures of mammalian cells capable of stimulating the production of titres of virus greater

than 10^3 TCD_{50} ml (Tables 17-19). When virus titres were lower than 10^3 TCD_{50} per ml haemadsorption did not occur.

Relation to CPE: The onset of changes in the infected cells associated with haemadsorption always followed a day or two after the onset of the early cytopathic changes such as vacuolization of the cytoplasm and fusion of cells. As with the onset of the CPE, the time at which haemadsorption was first detected was related linearly to the age of the infected cells (Fig. 6). The slopes of the two regressions, however, differed significantly ($F = 10.25^{**}$, d.f. 1, 173) such that the CPE always preceded haemadsorption but less so in young cells than in older cells.

Dose Effect: The onset of haemadsorption was related inversely to the dose of virus used to infect the cultures irrespective of the type of cell culture (Table 20). The relationships were linear and significant (Figs. 11 and 12).

Cell Effect: The onset of haemadsorption occurred significantly earlier in PMK cells than in PCK cells although the regressions between the dose of virus and the onset of haemadsorption were similar ($F = 0.01$, d.f. 1, 92) (Table 21; Fig. 11).

The onsets of haemadsorption in FL and LLC-MK2 cells were identical ($F = 0.25$, d.f. 1, 116) and, likewise, the regressions between dose of virus and

onset of haemadsorption were identical (F = 0.21, d.f. 1, 116) (Fig. 12). The onset of haemadsorption in Hep-2 cells was significantly slower than in the other two cell lines (F = 171.80**, d.f. 1, 176), and moreover, the regression of the onset on the dose of virus differed (F = 4.15*, d.f. 1, 176) (Fig. 12).

Haemadsorption in all but PCK cells was multifocal to start with and soon became diffuse. At the acme of CPE the entire monolayer was studded with erythrocytes. In PCK cells, however, haemadsorption was confined to small foci.

MEASLES HAEMAGGLUTININS

Replication of measles virus in primary monkey kidney cells and in established human and simian cell lines was accompanied by the production of haemagglutinins for simian erythrocytes. The haemagglutinins were first detected in the harvested fluids after the onset of the CPE and the haemagglutinin titres rose as the CPE increased (F = 24.39**, d.f. 1, 19) (Table 22). There was also a direct relationship between haemagglutinin and virus titres of the fluids provided the virus titres equalled or exceeded $10^{4.9}$ TCD_{50} per ml (F = 50.33**, d.f. 1, 3) (Table 23).

Evolution: Evolution of haemagglutinins was studied by infecting three days' old PMK and FL cells with $10^{4.6}$ to $10^{4.8}$ TCD_{50} per ml of the virulent Belfast and Holly Hill

strains of measles virus, and examining the pooled frozen and thawed harvests from batches of five tube cultures for haemagglutinins at different post-inoculation intervals (Tables 24-27; Figs. 13-16). Traces of haemagglutinins appeared on the sixth to eighth day after inoculation and reached detectable levels on the tenth day. Thereafter, the titres were stationary and were not influenced by the time and frequency of replenishment of the cell culture fluids. Titres of 1:4 or 1:8 per 0.2 ml were demonstrated from the eighth to eighteenth day. Decline of titres coincided with the disintegration of the cell sheets. There were no significant differences in the development of haemagglutinin titres in cultures infected with the virulent and adapted strains of measles virus (Table 28).

Effect of Age of Cells: There was no relationship between the age of FL and LLC-MK2 cells at the time of infection with the Holly Hill strain of measles virus and the yield of haemagglutinins ($F = 0.385$, d.f. 1, 26) (Table 29).

Dose Effect: The production of haemagglutinins was slightly and directly related to the dose of virus used to infect the cultures ($F = 6.45^{**}$, d.f. 1, 24) and was independent of the cell line used ($F = 0.08$ and $F = 0.03$, d.f. 2, 24) (Table 30).

Effect of Incubation Temperature: Variations in the temperature of incubation did not influence the

production of haemagglutinins (F = 0.34, d.f. 1, 13) (Table 31).

Effect of Actinomycin-D: Pre-treatment of FL cells with actinomycin-D had a salutary effect on haemagglutinin titres (t = 3.205**; P $\#<0.01$) (Table 32; Fig. 17). Apparently there was a concomitant increase in virus titres in the treated cultures but the values were not statistically significant (t = 0.726; P $\#>0.05$).

Cell-bound Haemagglutinins: Much of the haemagglutinins was cell-bound. In studies on the kinetics of haemagglutinin production in PMK, FL and LLC-MK2 cells infected with the Holly Hill strain, the cell-associated haemagglutinins were first detected on the fourth day in PMK cells and on the sixth day in the other two cell cultures. There was no evidence of release of haemagglutinins into the nutritive medium during this period. From the tenth day onwards, the titres of the cell-bound haemagglutinins were significantly higher than the titres of the released haemagglutinins (F = 127.43**; d.f. 1, 12) (Table 33; Fig. 18).

Cell disruption procedures such as freezing and thawing and sonication entailed the release of the cell-bound haemagglutinins. A minimum of two cycles of freezing and thawing and sonication for 3-5 minutes seemed sufficient. The efficiency of the two procedures was of the same order (t = 1.015; P $\#>0.05$) (Tables 34 and 35).

Effect of Tween 80: Whereas treatment of the crude, measles-infected culture harvests with ether had no effect on the haemagglutinin titres (Table 36), treatment with Tween 80 resulted in a 2 to 4-fold increase in titres; 10 mg/ml of the detergent were sufficient (Table 37).

Treatment with a combination of Tween 80 and ether resulted in a further 2-fold increase in the haemagglutinin titres. Treatment of the crude haemagglutinins with Tween 80 had to precede the treatment with ether. The reverse order of treatment did not affect the titres (Table 38). A ratio of two volumes of Tween 80 (10 mg/ml) to one volume of ether gave consistently high titres when the treatment was carried out at 4°C (Table 39).

Effect of Sodium Deoxycholate: Enhanced titres of haemagglutinins were also obtained after treatment of the crude haemagglutinins with sodium deoxycholate solution. Treatment with 25 to 75 mg/ml of the salt for a period of five minutes resulted in a 16 to 32-fold increase in haemagglutinin titres, the optimal concentration of the detergent being 50 mg/ml (Table 40).

Sodium deoxycholate treatment was four times more efficient than Tween 80 ether treatment (Table 41).

MEASLES COMPLEMENT-FIXATION ANTIGENS

Relation to Haemagglutinins: Nearly all the mammalian

cell cultures infected with measles virus strains contained complement-fixation (CF) antigens at the height of the CPE. The development of the CF antigens in measles cultures usually parallelled the development of the haemagglutinins (Tables 42-44). When the infected cell sheets were frozen and thawed or sonicated in the presence of the culture fluids and the harvests tested for haemagglutinins and CF antigens, the titres of both were found to be closely related. A notable exception was Vero cell cultures infected with the Holly Hill strain of the virus; there were traces of CF antigens but no haemagglutinins (Table 45). In fowl-embryo fibroblast cultures infected with the different adapted strains of the virus neither haemagglutinins nor CF antigens were demonstrable.

Relation to Infective Virus: Development of the CF antigens in measles cultures was related to the production of the infective virus particles but the presence of the CF antigens was not necessarily associated with high virus titres. For example, in its first passage in Vero cells the infectivity of the Holly Hill strain of measles virus was low but traces of CF antigens were found in the cell sheets. In the next two passages, although infectivity titres remained stationary the yield of the antigens improved; a titre of 1:4 was obtained from some harvests (Table 45). However, replication of the virulent Belfast strain in

the first two serial passages in Vero cells and up to five passages in PCK cells and of the Holly Hill, Beckenham and Colindale strains in MDCK cells was not accompanied by the production of CF antigens. The infectivity titres in these systems rarely exceeded 10^3 TCD_{50} per ml (Tables 42-45).

Host Cell Effect: In general, infected primary simian and human cell cultures gave significantly higher yields of CF antigens than the infected cell lines ($t = 3.947^{**}$ $P < 0.01$ and $t = 3.938^{**}$, $P < 0.01$ respectively) (Tables 42-45).

Virus Strain Differences: There were no differences in the titres of CF antigens in cultures infected with the Holly Hill and Colindale strains of measles virus ($t = 1.027$, $P > 0.05$) (Tables 42-44). Similarly, there were no differences in the titres of CF antigens in cultures infected with the virulent and adapted strains of measles virus ($t = 0.371$, $P > 0.05$) (Tables 42-44).

Effect of Incubation Temperature: Three days' old tube cultures of FL cells were infected with $10^{5.6}$ TCD_{50} per ml of the Holly Hill strain of the virus and batches of the cultures were held at 33, 36 and 39°C. At 6, 8 and 10 days post-inoculation, batches of five tubes from each group were frozen and thawed and the pooled fractions were tested for CF antigens. The titres on day six were 1:3, 1:4 and 1:2 per 0.1 ml in the cultures incubated at 33, 36 and 39°C respectively.

The titres increased two-fold on day eight. On the tenth day, the cultures incubated at 33 and 36°C had a titres of 1:8 whereas the cultures incubated at 39°C had a titre of 1:5.

Dose Effect: Five days' old bottle cultures of FL cells were seeded with $10^{5.6}$, $10^{4.6}$ and $10^{3.6}$ TCD_{50}/ml of the Holly Hill strain of measles virus. The nutritive medium was changed on the third and the sixth post-infection days. On days six and ten, three bottle cultures from each group were frozen and thawed and the pooled infective materials were titrated for CF antigens. The titres on day six in the cultures seeded with ten-fold dilutions of the virus were 1:5, 1:3 and 1:2 respectively per 0.1 ml. On day ten the levels were 1:8, 1:8 and 1:4 respectively. The data suggested that higher doses of the virus resulted in the infection of a higher percentage of cells and quicker laterial spread than the lower doses; statistically, however, the relationships were not significant ($F = 0.02$, d.f. 1, 7).

Evolution: Studies on the evolution of the CF antigens in LLC-MK2 and FL cells infected with the Holly Hill strain of measles virus revealed that, like haem-agglutinins, the CF antigens were largely cell-bound. Cell-associated antigens were first detected on the fourth day after infection. Thereafter, the titres of cell-bound and released antigens ran parallel until the

eighteenth day in FL cultures and the twentieth day in LLC-MK2 cultures; the difference between the two titres was 0.4 to 0.7 $\log_{10}$ units per ml (Fig. 19).

Actinomycin-D Treatment: In actinomycin-D treated FL cultures infected with $10^{4.8}TCD_{50}$ per ml of the Holly Hill strain of measles virus, CF antigens were detected as early as two days post-inoculation whereas in untreated cultures, the antigens were demonstrable two days later (Table 46). The differences in the developments of the CF antigens were not, however, statistically significant ($F = 3.25$, d.f. 2, 13) (Table 47).

Physical Aspects: Freezing and thawing and sonication were of equal efficiency in disrupting the cells to release the cell-associated antigens, (Tables 48 and 49). The antigens were precipitated from the tissue culture harvests by cold methanol and acetone, but both treatments lowered the titres (Table 50). On the other hand, precipitation with ammonium sulphate permitted a 70 per cent recovery of the antigens when infected harvests were treated to a final salt concentration of 35 per cent (Fig. 20).

Tissue Antigens: Suspensions of a specimen of measles-infected liver tissue were anti-complementary up to a dilution of 1:64. Fixation of the antigens with anti-measles rabbit serum was demonstrable in 1:80 but not higher dilutions. Inactivation of the suspensions at

55°C for 30 minutes or 60°C for 20 minutes did not influence the titres and the anti-complementary activity.

MEASLES IMMUNO-DIFFUSION ANTIGENS

Cell Culture Antigens:

Non-specific reactions: The titres of precipitating antigens obtained from measles-infected cell culture fluids by forced dialysis against "carbowax" rarely exceeded 1:4 per 0.1 ml. Moreover, the preparations contained non-viral diffusible antigens which reacted with a few rabbit anti-measles sera. Whereas adsorption of the sera with desiccated human liver powder or Hep-2 or FL cells did not abolish the non-specific immuno-diffusion reactions, adsorption overnight at 4°C with dried uninfected cell culture fluid that had been concentrated 500 times and freeze-dried was effective. The procedure, however, was tedious.

Physical aspects: Infected cell sheets that were disrupted by sonication or freezing and thawing yielded comparable titres of specific antigens (Table 51). On the other hand, precipitation with ammonium sulphate lowered the titres; a 40 per cent final concentration of the salt permitted 72 per cent recovery (Table 52).

Relation to CF antigens and haemagglutinins: The preparations containing immunodiffusion antigens exhibited varying degrees of complement-fixation and

haemagglutination activities; the mean ratio of the titres of the precipitating complement-fixing and haemagglutinating antigens were 1:8:10.

Tissue Antigens: The liver specimen from a case of measles showed the presence of immuno-diffusion antigens when diffused in agar-gels against the sera of rabbits inoculated with two doses of Behringwerke measles Tween-ether haemagglutinins or with one or two doses of live virus from PCK cell cultures (Fig. 21).

BEHAVIOUR OF DISTEMPER VIRUS IN CELL CULTURES

Virulent Strain:

Cytopathogenicity in primary cell cultures: On first isolation in PCK cells the three virulent strains of canine distemper virus induced CPE at 36°C, 14, 16 and 17 days respectively after inoculation (Table 53). Serial passage in PCK cells did not reduce the time of onset of the CPE. With two strains no evidence of virus replication was manifested after continued serial passage but the third isolate, No. 15/68, was carried successfully through five passages and designated the CTVM strain. In its fifth passage the CTVM strain was adapted to primary canine lung fibroblasts and canine lung macrophages. However, the virus could not be established in other primary mammalian cell cultures (Table 54).

In canine lung macrophage and fibroblast cultures

the CPE of the CTVM strain was manifested nine to ten days after infection. Serial passage did not reduce the time interval (Table 54). The degree of CPE was poor in both cell types.

Visual assessment of the CPE was unreliable and the characteristic cytomorphological features of distemper infection were more easily recognised in stained coverslip cultures.

Incubation temperature effect: The relationship between the temperature of incubation and the time of the onset of the CPE was studied by infecting 1-2 days old PCK cells with $10^{3.8}TCD_{50}$ per ml of the CTVM strain. Twelve observations were made at each temperature. As the temperature rose the time of onset of CPE decreased, the relationship being significant ($F = 9.35^{**}$, d.f. 1, 34) and linear (Fig. 22).

Effect of age of cells: The onset of CPE was shorter in one day old cells than in two day old cells (Table 55). The difference was statistically significant ($F = 1,288.55^{**}$, d.f. 1, 50). The responses to incubation temperature changes, however, were the same ($F = 1.79$, d.f. 1, 50) (Fig. 23).

Effect of animal sera: In cell cultures that were propagated on outgrowth media containing foetal calf serum and then maintained on media containing either foetal calf serum or bovine amniotic fluid, the CPE appeared earlier than in cultures that were grown and

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Effect of animal sera: In cell cultures that were propagated on outgrowth media containing foetal calf serum and then maintained on media containing either foetal calf serum or bovine amniotic fluid, the CPE appeared earlier than in cultures that were grown and

maintained on postnatal calf serum ($t = 9.453^{**}$, $P < 0.005$) (Table 56). Substitution of calf serum with dog serum conferred no advantage. In cultures that were propagated on media containing fowl, horse, rabbit or sheep sera the development of the CPE was delayed and its progress was poor. Even in cultures propagated and maintained on foetal calf serum the spread of the CPE was generally slow and marginal.

Cytopathogenicity in cell lines: In its seventh serial passage in PCK cells the virulent GTVM strain of canine distemper virus was adapted to MDCK cells. The virus did not grow in other mammalian cell lines (Table 57). Replication of the virus in MDCK cells was slow and poor and did not improve with serial passage.

Actinomycin-D treatment: Between the sixth and ninth serial passages the mean onset of CPE was shorter in cells pre-treated with actinomycin-D and the period was inversely related to the dose of the drug ($F = 137.36^{**}$, d.f. 1, 108) (Fig. 24).

Rockborn Strain:

Cytopathogenicity in primary cell cultures: The Rockborn strain of canine distemper virus replicated and induced CPE in canine cells only (Table 58). Serial passage tended to reduce the time of onset of the CPE particularly in PCK cells. Visual assessment of the CPE was difficult and the recognition of distemper-specific changes was only satisfactory in stained

preparations.

Effect of age of cells: There was a significant, direct, linear relationship between the time of onset of the CPE and the age at which PCK cells were infected with the Rockborn strain ($F = 281.20^{**}$, d.f. 1, 56) (Table 59; Fig. 25).

Incubation temperature effect: The relationships between the temperature of incubation and the time of onset of the CPE induced by the Rockborn strain in three day old PCK cells appeared to be inverse and approached linearity but the regression was not statistically significant ($F = 2.68$, d.f. 1, 34) (Fig. 26).

Actinomycin-D treatment: Pre-treatment of two day old PCK cells with actinomycin-D reduced the time of onset of the CPE and the period was inversely related to the dose of the drug ($F = 26.78^{**}$, d.f. 2, 48), the line of best fit being curvilinear ($F = 6.83^{**}$, d.f. 1, 47) (Fig. 27). The linear relationship was identical with that shown by treated PCK cells infected with the CTVM strain ($F = 1.02$, d.f. 1, 56) although the levels differed markedly ($F = 1917.86^{**}$, d.f. 1, 56).

Cytopathogenicity in cell lines: The Rockborn strain was inoculated into seven mammalian cell lines but induced CPE only in MDCK cells (Table 60). Both in onset and magnitude the overall CPE in MDCK cells was similar to that in PCK cells. Thus there was a significant direct relationship between the time of

onset of the CPE and the age at which MDCK cells were infected with the Rockborn strain ($F = 35.87^{**}$, d.f. 1, 76) (Table 61). Statistically, however, the regression in PCK and MDCK cells differed significantly but the age of the cells was more critical in PCK cultures than in MDCK cultures ($F = 111.92^{**}$, d.f. 1, 132) (Fig. 25). Similarly, the relationship between the temperature of incubation and the time of onset of the CPE in MDCK cells appeared to be inverse and approached linearity although the regression was not statistically significant ($F = 3.06$, d.f. 1, 34) (Fig. 26). A comparison of the behaviour of MDCK and PCK cells infected with the Rockborn strain confirmed the inverse relationship ($F = 5.22^{**}$, d.f. 1, 68), the slopes being similar ($F = 0.32$, d.f. 1, 68) but the levels differing significantly ($F = 93.44^{**}$, d.f. 1, 68). Likewise, pre-treatment of MDCK cells with actinomycin-D reduced the time of onset of the CPE and the period was inversely related to the dose of the drug ($F = 64.16^{**}$, d.f. 2, 47), the line of best fit being curvilinear ($F = 30.64^{**}$, d.f. 1, 47). The relationship, moreover, was identical with that shown by treated PCK cells infected with the Rockborn strain ($F = 0.72$, d.f. 2, 94) although the levels differed significantly ($F = 19.54^{**}$, d.f. 1, 94) (Fig. 27).

Onderstepoort Strain:

Cytopathogenicity in fowl embryo fibroblasts: The

cytopathic changes induced by the Onderstepoort strain of canine distemper virus were manifested between the third and fifth day after infection in fowl embryo fibroblast cultures. It was often difficult to differentiate the virus-induced cellular alterations from non-specific retrogressive changes associated with ageing of the fibroblasts particularly when the cells were four days of age or older at the time of infection.

Effect of age of embryos: There was a significant, direct, linear relationship ($F = 23.94^{**}$, d.f. 1, 18) between the mean period of onset of the CPE induced by the Onderstepoort strain and the age of the fowl embryos from which fibroblast cultures were prepared (Fig. 28).

Effect of incubation temperature: The time of onset of cytopathic changes in fowl embryo fibroblasts infected with the Onderstepoort strain of canine distemper virus was not influenced by the temperature at which the cultures were held ($F = 0.27$, d.f. 6. 93) (Table 62).

Effect of age of cells: As with the CTVM and Rockborn strains there was a significant, direct, linear relationship between the time of induction of the CPE and the age of the fowl embryo fibroblasts at the time of infection with the Onderstepoort strain of canine distemper virus ($F = 17.23^{**}$, d.f. 1, 58) (Fig. 29). This observation was confirmed by experiments designed to study the effect of the temperature of incubation on

the time of appearance of the CPE. At each temperature there existed a positive relationship between the age of the infected cells and the period of onset of the CPE; the relationship was linear and statistically significant when the cultures were incubated at 37 and 39°C (Table 63). Furthermore, the slopes and levels of the regressions were similar (F = 0.635 and F = 2.00, d.f. 2, 96).

Effect of dose of virus: The influence of the dose of virus on the development of cytopathic changes was studied by infecting cultures of fowl embryo fibroblasts with ten-fold dilutions of the Onderstepoort strain of canine distemper virus. A significant, inverse, linear relationship between the time of onset of the CPE and the amount of inoculated virus was found such that the CPE set in earlier with higher doses of the virus than with lower doses (F = 8.78**, d.f. 1, 28) (Fig. 30).

Actinomycin-D treatment: There were no differences in the time of onset of cytopathic changes induced by the Onderstepoort strain of canine distemper virus in cultures of fowl embryo fibroblasts pre-treated with actinomycin-D and in untreated cultures (F = 0.12, d.f. 1, 70) (Table 64).

Infectivity Titres:

Virulent strain: The infectivity titres of the CTVM strain in primary canine cells were significantly higher than the titres obtained in MDCK cells

($t = 5.163^{**}$; $P < 0.01$) (Table 65). Similarly, the titres in PCK cells were significantly higher than in primary canine lung fibroblast cultures ($t = 3.398^{*}$; $P < 0.05$).

A significant exhaltation of the infectivity titres occurred when MDCK cells were pre-treated with actinomycin-D and then infected with the CTVM strain, ($t = 3.011^{**}$; $P < 0.025$) (Table 66). Moreover, the increase in titres was apparently related to the dose of the drug, although the values were not statistically significant ($t = 2.128$; $P > 0.05$).

Adapted strains: The Rockborn strain of canine distemper virus induced significantly higher titres in MDCK cells than in PCK cells ($t = 3.439^{**}$, $P < 0.01$) (Table 65). The titres in either cell type were not related to the age at which the cells were infected ($F = 0.01$, d.f. 6, 3) (Table 67).

The assessment of infectivity of the Onderstepoort strain of canine distemper virus to fowl embryo fibroblast cultures was tedious. The titres apparently ranged from 5.22 to 5.88 (Table 65).

Cytomorphology:

Virulent strain in primary cell cultures: In general, the features of the CPE were similar to those induced by measles virus in PMK cells except that the changes developed much later (Fig. 31). After five serial passages the cytomorphological changes of the

infection in unstained PCK cells induced by the CTVM strain of canine distemper virus were recognized on the tenth or eleventh day post-inoculation. In stained cultures the first cytological evidence of the infection was manifested on the ninth day and was characterized by nuclear changes. The nuclei were eccentric and mitotic figures were scanty. The nucleoli fused to form a single central or paracentral hyperchromatic mass which was considerably enlarged and demarcated from the rest of the nuclear matrix by a distinct halo. A day later cytoplasmic abnormalities developed and were characterized chiefly by vacuolation, granularity and acidophilia. There were several binucleate and trinucleate cells and occasional uninucleate giant cells with enlarged hyperchromatic nuclei.

On the eleventh or twelfth day, well-marked syncytia were detected. These were polykaryocytes containing up to eight nuclei which were distributed either randomly or as a peripheral chain or as central cluster. Some nuclei in the syncytia contained a single, enlarged, hyperchromatic, basophilic nucleolar mass while others had inclusion bodies. The inclusion bodies sometimes occurred alongside the enlarged nucleolar mass from which they were differentiated by their characteristic acidophilia. Both the enlarged nucleolar mass and nuclear inclusion bodies were surrounded by an unstained halo.

Pari passu cytoplasmic inclusions were recognized in single cells and syncytia as multiple dot-like granules surrounded by a faint halo. Their tinctorial affinities were varied in their early stage of developments. In most syncytia the cytoplasmic inclusions were baso-acidophilic. Larger bodies showed distinct acidophilia which was better demonstrated in the phloxine-tartarazine method of staining. In acridine-orange stained preparations the inclusions were prominently apple-green against a background of varying shades of orange.

In unstained cultures the development of the syncytia was recognized on the eleventh or twelfth day. Stellate cells were detected a day or two later.

Host cell effect: The syncytia produced by the virulent CTVM strain in PCK cells and primary canine lung fibroblasts had similar cytological features. However, nuclear inclusions were scanty or difficult to recognize. The syncytia occurred much earlier, the fourth or fifth post-infection day in infected canine lung macrophage cultures but were small and scarce. They were differentiated from the syncytia of uninfected canine macrophage cultures by the presence of cytoplasmic and nuclear inclusions. Moreover, the syncytia in infected cultures were relatively large, numerous and contained more nuclei which were characteristically arranged like a string of pearls

(Fig. 32).

Incubation temperature effect: The syncytia were fewer and much smaller in size and the nuclear inclusions were difficult to recognize in cultures held at 33°C than in cultures incubated at 37 or 39°C.

Effect of animal sera: In infected cultures grown and maintained on media containing fowl, horse or sheep sera, the CPE was difficult to recognize. Syncytia were scenty and small in size, rarely containing up to eight nuclei. No cytoplasmic and nuclear inclusions were detected when sheep sera were used. With rabbit sera, the overall CPE was similar to that observed in cultures propagated and maintained on foetal calf serum, but its time of induction was protracted. Furthermore, stellate cells were predominant.

Virulent strain in MDCK cells: The main features of the CPE in MDCK cells infected with the virulent CTVM strain were similar to those observed in PCK cells except that nuclear inclusions were either absent or difficult to recognize.

Rockborn strain: The rapidity of the spread of the CPE induced by the Rockborn strain was a constant feature in cultures held at 37 and 39 than at 35°C. Moreover, in such cultures the syncytia were large and numerous and the nuclear and cytoplasmic inclusions were well-marked (Fig. 33). Complete desquamation of the cell sheet occurred on the tenth or eleventh day post-

inoculation when the cultures were infected with lower dilutions of the virus.

Dose effect: The dose of virus used to infect cultures was inversely and significantly related to the first appearance of syncytia, intracytoplasmic inclusions and intranuclear inclusions (Tables 68 and 69).

Onderstepoort strain: On the second or third day following inoculation with low dilutions of the Onderstepoort strain of canine distemper virus primary fowl embryo fibroblast cultures showed withdrawal of cytoplasmic processes, rounding off, clumping and shrinkage. Cytoplasmic granularity, acidophilia, vacuolation, eccentricity of the nuclei, pyknosis and hyperchromatism of the nuclear chromatin were concomitant features. These changes were not observed in uninfected fibroblasts of corresponding ages. Spread of the infection was rapid and was characterized by the development of multiple foci of rounded off, stellate or shrunken cells. The foci expanded and were necrotic on the fifth or sixth day. Massive desquamation of the cell sheet followed a day or two later (Fig. 34).

Dose effect: Cultures inoculated with higher dilutions of the virus often revealed the presence of large bizarre, fusiform cells containing up to six nuclei which were intensely hyperchromatic. No nuclear or cytoplasmic inclusions were detected in preparations stained by haematoxylin and eosin, phloxine-tartarazine

or acridine-orange.

Effect of animal sera: The use of animal sera in the maintenance media caused early onset of senile changes. Omission of serum from the maintenance medium facilitated differentiation of the specific from the non-specific cytological changes.

Other factors: The nature and severity of the cytological changes were not influenced by the temperature of incubation or the age of fibroblasts at the time in infection. Retrogressive changes associated with ageing generally developed on the sixth or seventh day after dispersion of the cells.

DISTEMPER COMPLEMENT-FIXATION ANTIGENS

Cell Culture Antigens: Infection of cell cultures with high doses of distemper virus resulted in the production of low titres of CF antigens which were detected at the acme of the CPE (Table 70). For antigen assays infected cell sheets and fluids were frozen and thawed thrice or sonicated and the suspensions clarified through low-speed centrifugation. Most CF antigens were cell-bound (Table 71).

Reaction specificity: Irrespective of the source, the cell culture antigens reacted only with anti-distemper sera. Similarly, sera containing distemper antibodies reacted specifically with distemper-infected cell culture fluids but not with uninfected culture

fluids (Table 72).

Physical aspects: The release of CF antigens from infected cell sheets through sonication or freezing and thawing was of equal efficiency ($F = 0.20$, d.f. 1, 15) (Tables 73 and 74). Three cycles of freezing and thawing followed by sonication for five minutes did not increase the yield of CF antigens (Table 75).

Relation to infective virus: There was no clear-cut link between infectivity and CF antigen titres ($F = 1.44$, d.f. 1, 6) (Table 76).

Host cell effect: The development of CF antigens was not apparently related to the host cell type. For example, there were no significant differences between the CF antigen titres of PCK and MDCK cell cultures infected with the Rockborn strain of virus (Table 77).

Virus strain differences: The strain of virus and its ability to propagate in a cell culture system influenced the yield of CF antigens. The CTVM strain, for example, grew poorly in MDCK cells and the CF titres induced were significantly lower than those induced by the same strain in PCK cells (Table 77). On the other hand, there were no significant differences between the CF antigen titres induced in PCK cultures by the CTVM and Rockborn strains ($t = 0.613$, $P > 0.05$).

Dose effect: There was a positive, significant, curvilinear relationship between the dose of virus,

Rockborn strain, and CF antigen production (F = 97.79**, d.f. 1, 26) which was not influenced by the host cell system (F = 1.11 and F = 2.32, d.f. 1, 26) (Fig. 35).

Evolution: MDCK and fowl embryo fibroblast cultures were respectively infected with the Rockborn and Onderstepoort strains of canine distemper virus using undiluted inocula. In MDCK cultures cell-bound CF antigens were first detected on the fourth post-infection day (Table 78). The antigen titres increased eight-fold to a peak about the tenth day. In contrast, the antigens released into the fluids were first detected on the sixth day and thereafter there was only a two-fold increase. The differences between the cell-associated and released antigen titres were highly significant ($t = 4.81^{**}$; $P < 0.01$).

The evolution of the CF antigens in fowl embryo fibroblasts infected with the Onderstepoort strain of canine distemper virus followed a similar pattern (Table 79). As before, the titres of the cell-bound antigens were significantly higher than the titres of the released antigens ($t = 7.94^{**}$; $P < 0.01$).

Actinomycin-D treatment: In fowl embryo fibroblasts pre-treated with 0.05 and 0.03 ug/ml of actinomycin-D and infected with the Onderstepoort strain of canine distemper virus, CF antigens were detected eight hours earlier than in untreated cultures (Table 80). Furthermore, from 8 to 120 hours

post-inoculation the CF antigen titres in the cultures treated with 0.05 and 0.03 ug/ml were similar ($t = 1.701$, $P > 0.05$) and were significantly higher than the titres in untreated cultures ($t = 12.880^{**}$; $P < 0.001$ and $t = 3.854^{**}$; $P < 0.01$ respectively). On the other hand, cultures pretreated with 0.01 ug/ml of actinomycin-D behaved like untreated cultures ($t = 0.230$; $P > 0.80$).

Tissue CF Antigens: Specific CF antigens were detected in 18 of 23 (61 per cent) sets of morbid tissues from clinical cases of alleged canine distemper (Table 81). Likewise, 4 of 11 sets of tissues from suspect cases of ferret distemper that had been stored at $-70^{\circ}C$ for 12 to 24 months revealed distemper CF activity (Table 82).

The antigen titres in canine lungs were significantly higher than in spleens ($t = 2.067^{*}$; $P < 0.05$). Titres in spleens were, likewise, significantly higher than the titres in lymph nodes ($t = 5.000^{**}$; $P < 0.001$) (Table 83). Brain tissue contained much less antigen. No significant differences were detected in the amounts of the CF antigens in the different tissues from sick dogs that died or were destroyed ($t = 0.129$; $P > 0.05$). Similarly, more CF antigens occurred in the spleens than in the lungs of infected ferrets ($t = 2.333^{*}$; $P < 0.05$) (Table 84).

Reaction specificity: Distemper-infected tissues

contained antigens which fixed complement in the presence of normal and anti-distemper sera. Non-specific fixation was unaffected by heating the tissue suspensions to 60°C for 30 minutes. However, non-specific fixation did not occur when four to five units of complement were used. On the other hand, suspensions of chorio-allantoic membranes of fowl embryos infected with the Onderstepoort strain of canine distemper virus and suspensions of the membranes from uninfected fowl embryos showed non-specific fixation that occurred even in the presence of four to six units of complement (Table 85). Exposure of the membrane suspensions to 60°C up to 60 minutes had no effect on non-specific fixation. Higher units of complement abolished the non-specific fixation, but also lowered the specific CF antigen titres 4 to 8-fold.

Physical aspects: Distemper CF antigens were precipitated from infected canine tissue suspensions by treatment with cold methanol, acetone, benzene and ammonium sulphate but treatment resulted in a loss of CF activity. The best recovery was obtained with ammonium sulphate using a final salt concentration of 35 per cent (Table 86). Precipitation with benzene was the least satisfactory method (Table 87).

DISTEMPER IMMUNO-DIFFUSION ANTIGENS

Cell Culture Antigens:

Reaction specificity: Traces of distemper immunodiffusion antigens were demonstrated in Roux flask cultures of MDCK and PCK cells and fowl embryo fibroblasts infected respectively with the Rockborn and Onderstepoort strain of canine distemper virus after the fluids had been concentrated approximately 250 to 500 times by forced dialysis against polyethene glycol. The preparations often reacted non-specifically with normal canine and rabbit sera. The antigens released from the infected cell sheets by sonication, however, contained specific CF antigens but the titres were poor. The titres were improved by precipitation of the sonicated suspensions with cold methanol or acetone or ammonium sulphate to a final salt concentration of 35 to 40 per cent. Even so, titres of the specific immunodiffusion antigens rarely exceeded 1:8 or 1:16 per 0.1 ml.

Relation to CF antigens: With sonicated infected cell sheets the ratio of the titres of ID to CF antigens ranged from 1:32 to 1:128 (Table 88).

Tissue antigens: Distemper-infected canine and ferret tissues were rich in specific ID antigens. Sixty-one and 45 per cent respectively of specimens of tissues from dogs and ferrets suspected to have died from distemper contained specific ID antigens (Tables 89 and 90).

Reaction specificity: Unlike the antigens in the

infected cell culture fluids, the ID antigens from infected canine and ferret tissues reacted with hyperimmune anti-distemper and anti-rinderpest sera but not with normal sera. Similarly, uninfected tissues did not react in gels with normal sera and hyperimmune anti-distemper and anti-rinderpest sera.

Distribution: Of the different canine tissues examined, the tonsillar tissues always contained specific ID antigens. The percentages of lymph nodes, lungs, spleen and brain specimens that were positive were 85, 78 and 50 respectively (Table 89). In respect of two clinical cases, ID antigens were demonstrated in lungs, spleen and lymph nodes but not in the brain. There were no differences in the distribution of ID activity in the tissues harvested from destroyed and dead animals.

The distribution of specific ID antigens in ferret lungs and spleens was of equal frequency and was not apparently affected by the length of storage of the tissues (Table 90). The antigens were also demonstrated in the liver and pancreas.

Relation to CF antigens: Infected tissues that contained specific CF antigens always yielded ID antigens but the titres of the ID antigens were always lower than the CF titres (Table 91). Thus the ratio of the titres of ID and CF antigens ranged from 1:32 to 1:64.

TEST PARAMETERS

MEASLES HAEMAGGLUTINATION TEST

Effect of Incubation Temperature: Auto-agglutination of simian erythrocytes frequently occurred when suspensions were incubated for 25 minutes or longer at temperatures above 42°C. To obviate overshadowing of measles haemagglutination (HA) virus-erythrocyte mixtures were first held either at 45°C for 15 minutes or at 50°C for 8 minutes and then at 42°C for a further 20 to 30 minutes.

Measles HA occurred at temperatures ranging from 4 to 50°C. Higher temperatures yielded faster results, the relationship between the logarithm of the time of onset of haemagglutination and the temperature being linear, negative and significant (Table 92; Fig. 36). The onsets of agglutination in plates and tubes were similar ($F = 0.80$ and $F = 1.40$; d.f. 1, 15). Optimal HA titres occurred at temperatures between 35 and 46°C (Table 93). Titres at 4°C were lower and were only reproducible when the reagents and containers were pre-chilled.

Similarly there was a direct relationship between temperature and the adsorption of haemagglutinins by 2.5 per cent erythrocytes (Table 94). At each temperature the rate of adsorption was linear and significant, the 50 per cent adsorption times (AT_{50}) varying from 353 minutes at 4°C to 10 minutes at 45°C

(Table 95). An Arrhenius plot of the rates was linear and significant ($F = 18.44^{*}$; d.f. 1, 3) suggesting that the reactions followed first-order kinetics (Fig. 37).

Effect of Cell Concentration: The relationship between the concentration of erythrocytes and HA titres was negative, curvilinear and significant ($F = 118.55^{**}$; d.f. 1, 31) (Fig. 38). High concentrations of erythrocytes removed haemagglutinins faster than low concentrations, the AT_{50s} using 2.5 and 5.0 per cent concentrations being 15 and 5 minutes respectively (Table 96; Fig. 39).

pH Effect: Haemagglutination only occurred at hydrogen ion concentrations between pH 4.0 to 10.5 (Table 97; Fig. 40). Optimal titres occurred between pH 6 and 9. Similarly, the optimal adsorption of haemagglutinins occurred between pH 6 and 9 (Table 98).

Diluent Effect: The type of saline diluent had no influence on the HA titre (Table 99). The concentration of sodium chloride in the diluent was, however, critical (Table 100). Molar concentrations of 0.40 or higher caused flocculation, auto-agglutination and haemolysis. Calcium and magnesium ions were also essential, the optimal molar concentrations being respectively 0.03-0.003 and 0.05-0.005 (Table 101).

Elution: The HA reaction was irreversible. No elution of virus occurred despite prolonged incubation at $4^{\circ}C$ to $50^{\circ}C$ at pH values ranging from 4 to 10.

Neuraminidase-treatment (0.15 to 0.5 ug/ml) of the erythrocyte suspensions before and after adsorption with the virus did not result in the elution of the virus.

Haemolysis: Prolonged incubation of the virus-erythrocyte mixtures at temperatures above 37°C resulted in partial haemolysis of the agglutinated erythrocytes when the pH ranged from 5 to 10. Both neuraminidase-treated and formolized suspensions underwent incomplete haemolysis.

Red Cell Spectrum: Measles virus and haemagglutinins agglutinated simian erythrocytes only. The highest titres were obtained with baboon erythrocytes and the lowest with rhesus and talapoin erythrocytes (Table 102). Suspensions of washed simian erythrocytes stored at 4°C were usable for at least three months.

Suspensions of mammalian erythrocytes from cats, cattle, dogs, ferrets, goats, guinea pigs, horses, human types O, A and B, mice, pigs, rabbits, rats and sheep were not agglutinated over the temperature range 4 to 37°C and pH range 5 to 10. Similarly, suspensions of avian erythrocytes from ducks, fowls, fowl embryos, and geese and suspensions of frog erythrocytes were unaffected. The tests were all replicated five times. Moreover, Behringwerke Tween-ether haemagglutinins were not adsorbed when incubated with 5 per cent suspensions of the different erythrocytes at different temperatures in the pH range 6 to 9.

Tanned Erythrocytes: The sensitivity of tannic acid-treated baboon erythrocytes was similar or marginally greater than that of the untreated erythrocytes (Table 103). Moreover, the treatment gave reproducible titres only with freshly collected blood samples. Stored samples of blood tended to auto-agglutinate or haemolyse.

Neuraminidase-treated Erythrocytes: Monkey erythrocytes pre-treated with neuraminidase were two to four times more reactive than untreated erythrocytes, the effect being dose, temperature and time-dependent such that higher doses, higher temperatures and longer treatment times yielded higher HA titres (Table 104). The origin of the neuraminidase-treated erythrocytes did not influence the rates of the reactions ($F = 0.38$; d.f. 5, 18) (Table 105). Rhesus and cynomologus erythrocytes behaved similarly ($F = 0.89$; d.f. 3, 12) and HA titres with them were significantly lower than titres with baboon erythrocytes ($F = 45.15^{**}$; d.f. 1, 26) (Table 106; Fig. 41).

Neuraminidase-treated erythrocytes from cattle, fowls, geese, guinea pigs, horses, rabbits and sheep were not agglutinated by measles Tween-ether haem-agglutinins. Likewise, three samples of human A and B blood group erythrocytes were refractory. On the other hand, three of five samples of human O group erythrocytes were agglutinated to a titre of 1:2 or 1:4 by Tween-ether and deoxycholate-treated haemagglutinins

but not by crude tissue culture haemagglutinins. The specificity of the reaction could not be determined unequivocally by haemagglutination-inhibition tests because of the low HA titres.

Effect of Formalin: Treatment of monkey erythrocytes with a final concentration of up to 1 per cent formalin did not alter their sensitivity to agglutination by measles virus or haemagglutinins. However, when fresh or stored erythrocytes were treated with higher concentrations of formalin, there was a progressive fall in the HA titres (Table 107). The relationships were linear, negative and significant and were independent of whether or not the erythrocytes were fresh or stored ($F = 1.89$ and $F = 2.43$, d.f. 1, 11) (Table 108; Fig. 42). Nevertheless, fresh blood samples were preferable to stored samples because stored samples were more prone to haemolyse (Table 109). On the other hand, formalinization stabilised erythrocyte suspensions.

Haemolysis occurred both during formalin-treatment and during centrifugation of the formalinized cells to remove residual formalin. The temperature at which formalinization occurred was critical such that higher temperatures induced haemolysis in a higher percentage of samples (Table 110). Haemolysis was not arrested by the addition of bovine serum albumin or baboon serum to the erythrocyte suspensions.

Confirmation of the finding that the reactivity of

baboon erythrocyte receptors with measles haem-agglutinins was not affected by low concentrations of formalin was obtained by adsorption tests with baboon erythrocyte and erythrocyte membrane suspensions. The propensity of Behringwerke Tween-ether haemagglutinins to agglutinate baboon erythrocytes was selectively inhibited by pre-incubation with baboon erythrocyte membranes at 37°C for an hour. The supernatant fluids obtained after centrifugation of the haemagglutinin-erythrocyte membrane mixtures contained no residual haemagglutinins when tested against baboon erythrocytes (Table 111). Treatment at 37°C of the suspensions of erythrocytes or erythrocyte membranes with different concentrations of formalin, ranging from 1 to 10 per cent, resulted in a progressive loss of the ability to adsorb haemagglutinins (Table 112). The relationship between the concentration of formalin and the residual HA titres was linear, positive and significant (Table 113). Moreover, formalinized whole erythrocytes and formalinized erythrocyte membranes behaved similarly ($F = 1.03$ and $F = 2.93$, d.f. 1, 42).

Effect of Formalin on the Haemagglutinins: To rule out the possibility that the occurrence of the gradual reduction in haemagglutination titres of baboon erythrocyte suspensions following exposure to higher concentrations of formalin was due to the direct action of residual formalin in the erythrocytes on the

haemagglutinins, studies were carried out to determine the effect of formalin on Tween-ether haemagglutinins. Inactivation by increasing concentrations of formalin was linear and significant (Fig. 43). The haemagglutinins, however, survived significantly higher concentrations of formalin than the receptors on the erythrocytes and erythrocyte membranes ($F = 34.81^{**}$; d.f. 1, 41). The reactions were also time-dependent (Table 114), the inactivation being linear and significant (Table 115). The half-life values at 37°C when 1.25 and 2.5 per cent concentration of formalin were used were similar ($F = 2.11$, d.f. 1, 20) and differed very significantly from the half-life value when 5.0 per cent formalin was used ($F = 214.05^{**}$; d.f. 1, 26).

Trypsin Inactivation of the Haemagglutinins: Incubation at 37°C of measles Tween-ether haemagglutinins with concentrations of trypsin ranging from 10 to 1000 ug/ml caused degradation (Table 116). The inactivation rates were all linear and significant (Table 117) and a plot of the half-life values at the various concentrations of the enzyme revealed a linear, negative and significant relationship suggesting a first order reaction (Fig. 44).

Trypsin inactivation of measles Tween-ether haemagglutinins was also influenced by the hydrogen ion concentration (Table 118). At each hydrogen ion concentration between pH 5 and 10 the inactivation was linear and significant (Table 119). A plot of the

half-life values revealed a curvilinear relationship, the nadir of which occurred at pH 8.4 (Fig. 45). In other words, inactivation was most rapid at pH 8.4.

Trypsin also inactivated the receptors on baboon erythrocytes (Table 120). The relationships approached linearity and significance (Table 121) and higher concentrations of trypsin inactivated more quickly than lower concentrations, the half-life values for 10 and 100 µg/ml being respectively 5.9 and 3.6 minutes. Trypsin inactivation of the erythrocyte receptors was significantly faster than trypsin inactivation of the haemagglutinins (Table 122).

Effect of Oxidizing Agents: The action of oxidizing agents on Behringwerke measles Tween-ether haemagglutinins varied; hydrogen peroxide had no effect; potassium permanganate and potassium perodate depressed the HA titres when relatively high molar concentrations were used, whereas iodine was active even at low concentrations (Table 123).

Unlike haemagglutinins, the receptors on baboon erythrocytes were not adversely affected by oxidizing agents (Table 124).

Thermostabiligy of the Haemagglutinins: Measles Tween-ether, deoxycholate-treated and crude untreated haemagglutinins had similar thermal decay patterns (Tables 125-129). At each temperature, inactivation was linear and usually significant (Tables 130-134).

There were, however, dramatic differences in the half-life values between haemagglutinin preparations (Table 135). Arrhenius plots of the thermal inactivation of the different preparations yielded linear regressions that were significant or approached significance (Table 136). Statistically, the Arrhenius plots of the Burroughs Wellcome preparation and the three CTVM preparations were identical ($F = 0.05$ and $F = 1.00$; d.f. 3, 8) and differed significantly in level but not in slope from the Arrhenius plot of the Behringwerke Tween-ether preparation ($F = 70.38^{**}$ and $F = 2.38$ respectively; d.f. 1, 16) (Fig. 46). In other words, the Behringwerke Tween-ether preparation was much more thermostable than the other preparations. Exposure of Behringwerke measles haemagglutinins to higher temperatures entailed faster inactivation, the rate of inactivation at each temperature being linear and significant (Tables 137-138; Fig. 47). The half-life values ranged from 10 minutes at 55°C to 2-5 minutes at 65°C.

Effect of Lyophilization: Five batches of Behringwerke measles tween-ether haemagglutinins were separately lyophilized and the ampoules were sealed under vacuum. The HA titres were assessed before and after treatment, each batch being replicated five times (Table 139). HA activity was not destroyed by lyophilization although the difference between the titres before and after

treatment was significant, the fall in titre being 0.25 ($t = 2.810^{**}$, P 0.01).

pH Stability of the Haemagglutinins: Behringwerke Tween-ether haemagglutinins stored at -10°C at different hydrogen ion concentrations were stable over the pH range 6 to 9 for at least 26 weeks, the longest period tested (Table 140). At higher and lower pH values significant linear inactivation occurred (Table 141; Fig. 48).

Adsorption of the Haemagglutinins by Red Cell Membranes: A suspension of baboon erythrocyte membranes having an adsorption titre of 2.8 $\log_{10}$ units per ml was mixed in equal volumes with measles Tween-ether haemagglutinins. The mixture was distributed in small aliquots and incubated at 22, 30 or 37°C for different periods. The residual HA titres were determined.

The rates of adsorption of the haemagglutinins by baboon red cell membranes were linear and significant (Tables 142-143). The rates appeared to be influenced by the temperature of incubation such that the adsorption was fastest at the highest temperature; the Arrhenius plot, however, was not significant ($F = 0.72$, d.f. 1, 2).

Thermostability of Red Cell Membranes: The ability of baboon erythrocyte membranes to adsorb measles Tween-ether haemagglutinins was reduced exponetially by exposure to 50 or 55°C (Table 144). At each temperature inactivation was linear and significant,

the half-life values being respectively 23 and 12 minutes (Fig. 49).

MEASLES HAEMAGGLUTINATION-INHIBITION TEST

Reaction Specificity: Measles haemagglutination was prevented by anti-measles serum irrespective of source (Table 145). The specificity of the reaction was not restricted to measles antibodies; anti-rinderpest sera from different species, some samples of anti-distemper sera from dogs and sera from two goats that were exposed to a natural attack of "peste des petits ruminants" inhibited the reaction. On the other hand, sera containing neutralising antibodies for human influenza types A, B and C, fowl plague, Newcastle disease, mumps, parainfluenza types 1, 2, 3 (human and bovine) and 4 and respiratory syncytial viruses failed to inhibit the reaction.

Non-Specific Inhibition: Non-specific inhibitors of measles haemagglutinins were detected in 44, 28, 17 and 25 per cent of cat, dog, ferret and rat sera (Table 146) but not in the sera of 203 cattle, 25 fowls, 32 goats, 20 horses, 17 monkeys, 96 pigs, 43 rabbits and 80 sheep.

Pre-treatment with a 25 per cent suspension of kaolin in PBS (ph 7.2) or acetone or a heparin-manganous chloride mixture effectively removed the non-specific inhibitors from animal sera. Kaolin treatment was simple and easy to perform; irrespective of

were also used for adsorption of the heterologous agglutinins from animal sera. The use of high titre simian red cell membrane suspensions was, however, frought with disadvantages. When animal sera having low titres of non-specific agglutinins for monkey erythrocytes were incubated with high titre red cell membrane suspensions and then centrifuged, the supernatant fluids contained good concentrations of unreacted red cell membranes. When such sera were later tested for haemagglutination-inhibition antibodies, spurious HI titres resulted due to the adsorption by the red cell membranes of the added haemagglutinins. This artefact was prevented by inactivation of the adsorbed sera at 60°C for 20 minutes before running the HI test.

Effect of Antigen Concentration: Block titrations in triplicate were carried out using descending two-fold dilutions of rabbit and calf anti-measles sera samples and ascending two-fold dilutions of Behringwerke Tween-ether haemagglutinins. The mixtures were held at 22°C for two hours and 0.5 per cent baboon erythrocytes were added. The relationships between the HI titres of the sera and the doses of antigen were linear, negative and statistically highly significant (Tables 152 and 153; Fig. 51). In other words, high doses of antigen depressed the HI titres. Whereas the slopes of the regression lines were similar ($F = 1.100$; d.f. 4, 74), the levels were statistically different ($F = 139.79^{**}$;

d.f. 4, 74).

Onset of HI: The onset of the inhibition of measles HA by anti-measles sera was directly influenced by the time of incubation of the serum-antigen mixtures (Fig. 52). The relationship was linear, positive and highly significant (Table 154). The source of serum did not affect the relationship ($F = 2.02$; d.f. 2, 21) although the antibody levels differed significantly ($F = 199.19^{**}$; d.f. 2, 21).

Effect of the Temperature of Incubation: Irrespective of the source, the HI titres of sera were similar when tests were run at 4, 22 and 37°C (Table 155).

Quality of Antigen: Whatever the source of antibodies, HI titres were significantly influenzed by the source of antigen ($F = 15.01^{**}$; d.f. 1, 22) (Table 156). Burroughs Wellcome haemagglutinins and CTVM crude haemagglutinins gave similar HI titres ($F = 0.25$; d.f. 1, 14) which were seven-fold lower than the HI titres with Behringwerke Tween-ether haemagglutinins, a difference statistically highly significant ($t = 5.459^{**}$; $P < 0.01$). Moreover, low titres of antibodies were only detected by testing with Behringwerke Tween-ether haemagglutinins (Table 157).

The efficiencies of CTVM Tween-ether and deoxycholate-treated haemagglutinins were similar to that of Behringwerke Tween-ether haemagglutinins ($F = 0.01$; d.f. 2, 27) (Table 158).

Thermostability: Measles haemagglutination-inhibition antibodies were unaffected by exposure to temperatures up to 65°C (Table 159). At 70°C denaturation occurred; the reactions were linear and significant (Table 160; Fig. 53). The rate of the denaturation was not influenced by the source of antibody ($F = 0.04$; d.f. 3, 14), although the antibody levels differed significantly ($F = 24.37^{**}$; d.f. 3, 14). Incorporation of bovine serum albumin to a concentration of 5 per cent had no effect on the denaturation ($F = 0.44$; d.f. 1, 18).

MEASLES HAEMADSORPTION TEST

In the main, the parameters of the measles haemadsorption test were similar to the parameters of the measles haemagglutination test.

Effect of Incubation Temperature: Visual assessment of haemadsorption was subjective. However, the effect of the temperature of incubation on the time of onset and magnitude of haemadsorption was clear cut; in cultures tested at 37 and 22°C haemadsorption developed after 20 and 30 minutes respectively, whereas at 4°C it developed later than 60 minutes (Table 161). Further, haemadsorption at 37°C was followed by partial haemolysis if incubation was continued for 4 to 5 hours. On the other hand, partial haemolysis was never detected in cultures that were held at 22 and 4°C for 72 hours.

pH Effect: Haemadsorption occurred with equal facility over a pH range of 4 to 10 (Table 162). Partial haemolysis occurred with prolonged incubation of the cultures and was readily recognized in hydrogen ion concentrations ranging from 6 to 9 units.

Reaction Specificity: Haemadsorption of simian erythrocytes was specific and irreversible in measles-infected cell cultures. Haemadsorption never occurred in uninfected cultures. Rhesus, cynomologous and baboon erythrocytes that were agglutinated by measles haemagglutinins were not adsorbed to measles-infected cells, whereas suspensions of rhesus and baboon erythrocytes that were pre-treated with human influenze type A and Newcastle disease haemagglutinins or with canine distemper virus were adsorbed.

Non-Specific Haemadsorption: Erythrocytes from cattle, dogs, goats, guinea pigs, hamsters, horses, mice, pigs, rabbits, rats and sheep were not adsorbed to measles-infected cells in the pH range of 5 to 10 at temperatures of 4, 22 and 36°C. Erythrocytes from avian species such as ducks, fowls, and geese were also refractory. With a few samples of frog erythrocytes there was non-specific haemadsorption by infected and uninfected LLC-MK2 cell cultures at 4 and 22°C in the pH range of 6 to 9. With infected cultures, the non-specific haemadsorption was not prevented by anti-measles serum.

Effect of Neuraminidase: Treatment of measles-infected cells with 0.15 to 0.30 mg per ml of neuraminidase or the use of neuraminidase-treated (o.15 mg per ml) baboon erythrocytes had neither salutary nor inhibitory effect on measles haemadsorption. Further, the enzyme treatment did not alter the refractoriness of erythrocyte suspensions from cattle, dogs, fowls, fowl embryos, geese, goats, guinea pigs, hamsters, horses, man, pigs, rabbits, rats and sheep to haemadsorption by measles-infected cells.

Formalinized and tannic acid-treated baboon erythrocytes were adsorbed to measles-infected cells.

MEASLES HAEMADSORPTION-INHIBITION TEST

Temperature of Incubation: Irrespective of the source, inhibition of haemadsorption of baboon erythrocytes by measles antibody occurred with equal efficiency at 22 and 37°C after incubation for one hour ($t = 1.024$; $P > 0.05$) (Table 163).

Time of Incubation: The efficiency of haemadsorption-inhibition by measles antibody increased with incubation time (Table 164). The rates were linear and significant (Table 165) and independent of the temperature of incubation ($F = 0.03$ and $F = 0.38$, d.f. 1, 8).

MEASLES NEUTRALIZATION TEST

Thermal Decay of Measles Virus: The parameters of the thermal inactivation of measles virus were ascertained for the temperatures commonly used in serological tests before studying the dynamics of the neutralization test (Table 166). At each temperature the decline in the virus titre was linear and significant and the half-life values ranged from 14 hours at 4°C to 5 hours at 37°C (Table 167). An Arrhenius plot of the thermal inactivation rates was linear and significant ($r = -0.999^*$) (Fig. 54).

Incubation Temperature Effect: Two-fold dilutions of anti-measles sera of human, simian and rabbit origin were incubated with a constant concentration of virus for one hour at 37°C, two hours at 25°C and 18-20 hours at 4°C and then the serum-virus mixtures were inoculated into FL cell cultures. The titres of the mixtures held at 37°C were about 0.3 $\log_{10}$ units higher than the titres of the mixtures held at 4°C; the titres of the mixtures held at 25°C occupied an intermediate position (Table 168).

Incubation Time Effect: Neutralizing antibody titres progressively increased with lengthening of the time of incubation; the relationship was linear, positive and significant (Fig. 55).

Virus Dose Effect: Neutralising antibody titres were related inversely to the amount of virus in the serum-

virus mixtures. The relationship was linear, negative and significant such that a two-fold increase in the amount of virus depressed the antibody titre by 0.2 $\log_{10}$ units (Fig. 56).

DISTEMPER NEUTRALIZATION TEST

The parameters of the distemper virus-serum neutralization test were determined by methods similar to those used in examining measles neutralization test. Thermal Decay of Distemper Virus: The inactivation of the Rockborn strain of canine distemper virus was studied at 4, 25 and 36°C. The half-life values ranged from 11 hours at 4°C to 3 hours at 37°C (Table 169). At each temperature the reduction in the virus titre was linear and significant and an Arrhenius plot of the thermal inactivation rates was linear with time and significant (Table 170; Fig. 57). Incubation Temperature Effect: Two-fold dilutions of rabbit anti-distemper sera were incubated with $10^{3.4}$ TCD_{50} of the Rockborn strain of canine distemper virus for 2 hours at 36°C, 2 hours at 25°C and 18-20 hours at 4°C. The serum-virus mixtures were then inoculated into MDCK cell cultures. With increase of incubation temperature, there were increases in the neutralising antibody titres which were collectively positive, linear and significant ($F = 36.52^{**}$, d.f. 1, 3), the slopes and levels being similar ($F = 0.43$ and

F = 17.00*, d.f. 2, 3) (Fig. 58).

Incubation Time Effect: Rabbit and dog anti-distemper sera were examined for neutralising antibodies by incubating ten-fold dilutions of the sera with $10^{3.4}$ TCD_{50} of the Rockborn strain of canine distemper virus at 25°C. With prolongation of the incubation period there was a progressive increase in the antibody titres, the relationships being positive, linear and significant (F = 152.56**, d.f. 1, 6). The slopes of the two regression lines were identical (F = 4.44, d.f. 1, 6) but the levels differed markedly (F = 137.21**, d.f. 1, 6) (Fig. 59).

Virus Dose Effect: The dose of the Rockborn strain of canine distemper virus influenced the neutralising antibody titres of rabbit and dog anti-distemper sera, the relationship being negative, linear and significant (F = 157.14**, d.f. 1, 6) (Tables 171 and 172). The regression slopes were identical (F = 2.78, d.f. 1, 6) such that an increase of 1 $\log_{10}$ unit of the virus resulted in the decrease of 1 to 1.3 $\log_{10}$ units of the antibody levels. As before, there was a significant difference in the levels of the regressions (F = 19.60**, d.f. 1, 6).

MEASLES COMPLEMENT FIXATION TEST

Effect of Incubation Temperature: Human and rhesus monkey sera were tested for CF antibody titres by

incubating the antigen-serum mixtures at 4, 22, 30 and 37°C; four units of antigen and two full units of complement were used. Irrespective of the source of serum ($F = 0.48$; d.f. 1, 20), the CF titres were related inversely to the temperature of incubation such that the titres at 4°C were 16 to 32-fold higher than at 37°C (Fig. 60). The CF titres of the rhesus monkey serum were significantly lower than the titres of the human serum ($F = 6.67^{**}$; d.f. 1, 20).

Effect of Antigen Concentration: Sera from four species were tested in quadruplicate using 16, 8, 4 and 2 units of antigen. Two full units of complement were added and the mixtures were held at 4°C for 18-20 hours. Irrespective of the source of antibody, best titres were obtained with 4 units of antigen (Table 173).

Effect of Complement Concentration: That the number of units of complement incorporated in the test had a profound influence on measles CF antibody titres was evident when samples of sera from three species were examined. Four units of antigen were used and the mixtures were incubated at 4°C overnight. There was a significant inverse linear relationship between the CF antibody titres and the concentration of complement (Table 174; Fig. 61) irrespective of the source of antibody ($F = 0.43$; d.f. 2, 48). Moreover, the source of antibody significantly influenced the titres ($F = 57.29^{**}$; d.f. 2, 48).

DISTEMPER COMPLEMENT-FIXATION TEST

Incubation Temperature Effect: Rabbit, calf and dog anti-distemper sera were titrated for CF antibodies using 4 and 2 units respectively of antigen and complement. The antigen-serum mixtures were held at 4°C for 18-20 hours and at 22, 30 and 37°C for 30 minutes. At each temperature the test was replicated.

Irrespective of the source of serum ($F = 0.97$, d.f. 2, 26) there existed, a significant, inverse, linear relationship between the temperature of incubation and the antibody titres ($F = 172.90^{**}$, d.f. 1, 26) (Fig. 62). With rabbit and dog sera the titres at 4°C were 1.4 to 1.6 $\log_{10}$ units higher than the titres at 37°C (Table 175). Comparison of the three regression lines revealed marked differences in levels ($F = 111.48^{**}$, d.f. 2, 26), the calf serum being the lowest.

Effect of Complement Concentration: To study the influence of the amount of complement on the distemper CF antibody titres, two-fold dilutions of rabbit and dog anti-distemper sera were incubated at 4°C for 18-20 hours with 4 units of antigen and varying amounts of complement. The relationship between the CF antibody titres and the number of units of complement used was inverse, linear and significant ($F = 442.11^{**}$, d.f. 1, 20) such that higher amounts of complement depressed the titres (Fig. 63). Furthermore, the source of the antibody significantly influenced the levels of the regressions ($F = 23.69^{**}$, d.f. 1, 20).

Effect of Antigen Concentration: Calf, dog and rabbit anti-distemper sera were tested using 16, 8, 4, 2 and 1 units of antigen. Two units of complement were then added and the mixtures were incubated for 18 hours at 4°C. The tests were replicated three times.

Optimal CF titres were obtained with 4 units of the antigen irrespective of the source of serum (Table 176).

MEASLES IMMUNODIFFUSION TEST

Effect of Incubation Temperature: The appearance of measles antigen-antibody precipitations in agar-gels was dependent on time and temperature of exposure. When Behringwerke Tween-ether haemagglutinins were diffused against antisera prepared against the haemagglutinins, a single line of precipitation emerged at 16, 16 and 48 hours respectively at 4, 22 and 37°C (Table 177; Fig. 64). With prolongation of the incubation period, the number of precipitation lines increased. The reaction was complete with the development of three precipitation lines and the speed of the reaction was related directly and significantly to the incubation temperature ($F = 8.64^{*}$; d.f. 1, 7). Likewise, the reaction was significantly faster at 37 than at 22 and 4°C when cell culture virus was diffused against viral antibodies ($F = 20.64^{**}$; d.f. 1, 7). Prolongation of incubation at 37°C often resulted in the fading or extinction of one, or sometimes, two precipitation lines. However,

at 22°C the lines persisted up to 351 days, the longest period observed.

Effect of Antigen Concentration: When constant amounts of serum (2.8 $\log_{10}$ per ml) were diffused against two-fold dilutions of Behringwerke Tween-ether haemagglutinins a positive relationship was detected between antigen concentration and the rate of development and magnitude of the immunodiffusion reaction (Table 178). The relationship approached linearity ($F = 16.22$, d.f. 1, 2).

Effect of Antibody Concentration: A similar, positive relationship existed between antibody concentration and the appearance and degree of immunodiffusion reaction (Table 179). The rate of reaction approached linearity ($F = 11.57$, d.f. 1, 2). A constant amount of Tween-ether haemagglutinins (2.8 $\log_{10}$ per ml) was used.

Effect of Heat: Sera of rabbits inoculated with two doses of either measles cell culture virus or heat-inactivated haemagglutinins or Tween-ether haemagglutinins produced three distinct lines of precipitation when diffused against the Behringwerke Tween-ether haemagglutinins. Further, when two-fold dilutions of Tween-ether haemagglutinins were allowed to react with an optimal dilution (2.2 $\log_{10}$ per ml) of the antibody, the three precipitation lines were clearly separated from one another. Exposure of the Behringwerke Tween-ether haemagglutinins and the cell

culture virus to 60°C for 30 minutes entailed the disappearance of one of the fast moving precipitation bands with a concomitment reduction in the titres of the haemagglutinins and the virus (Table 180).

Effect of Sodium Chloride: Different molar concentrations of sodium chloride were incorporated in the agar gel and two-fold dilutions of the Behringwerke Tween-ether haemagglutinins were diffused against an optimal dilution of the antibody with the objective of achieving a better delineation of the precipitation lines. There was no difference either in the overall pattern of diffusion or in the number of precipitation lines in tests conducted with untreated gels and gels impregnated with sodium chloride up to a concentration of 0.02 M. Higher concentrations increased the opacity of the gels impairing visual assessment and also reduced the number of precipitation lines and antigen titres (Table 181).

Reaction Specificity: Antibodies that were adsorbed with uninfected sonicated monkey cells produced the same number of precipitation lines against Tween-ether haemagglutinins as unadsorbed antibodies. However, adsorption of antisera prepared against cell culture virus with concentrated, freeze-dried, uninfected cell culture medium resulted in the removal of one of the precipitation lines when the sera were diffused against the virus.

DISTEMPER IMMUNO-DIFFUSION TEST

Effect of Incubation Temperature: To study the effect of incubation temperature on the rate of distemper immuno-diffusion, a preparation of distemper tissue ID antigens having a titre of 2.5 $\log_{10}$ units per 0.1 ml was diffused through agar against a specimen of dog anti-distemper serum. Five plates were used to study the influence of three different temperatures, namely, 4, 22 and 37°C.

The speed of immuno-diffusion reaction between distemper ID antigens and antibodies was directly related to the incubation temperature such that the appearance of the precipitation was significantly faster at 37 than at 4°C ($F = 5.71^{*}$; d.f. 1,12) (Table 182; Fig. 65). However, irrespective of the temperature of incubation, the precipitation line generally developed closer to the antibody wells than the antigen wells (Fig. 66).

Effect of Antigen Concentration: The influence of the concentration of the antigens on the speed of distemper immuno-diffusion was studied by diffusing different dilutions of the tissue antigens against a constant amount (2.0 $\log_{10}$ units per 0.1 ml) of the antiserum. The test was replicated three times.

The relationship between antigen concentration and the development of precipitation was positive, linear and significant ($F = 14.91^{**}$, d.f. 1, 7) (Table 183;

Fig. 68). With four to eight units of antigen the precipitation line developed at an optimal distance between the reactant wells (Fig. 67).

Effect of Antibody Concentration: As with antigens, the rate of distemper immuno-diffusion was directly and significantly related to the concentration of antibodies ($F = 8.59^*$, d.f. 1, 7) (Table 184; Fig. 68). A constant amount of the antigens (1.9 $\log_{10}$ units per 0.1 ml) was used in the tests.

Comparison of the effects of the concentration of the antigens and antibodies on the appearance of precipitation lines showed that the slopes and levels of the two regressions were similar ($F = 0.13$ and $F = 0.08$ respectively; d.f. 1, 14).

Effect of Heat: Distemper cell culture and tissue immuno-diffusion antigens were thermostable at 60°C for 30 minutes in a pH range of 5 to 9. However, there was a slight drop in the titres of the antigens after heat treatment (Table 185).

MEASLES ANTIBODIES

MAN

Sera from 15 babies and 15 adults of different ages were examined for measles antibodies by HI and CF tests; parallel neutralization tests (N) were run with a few sera.

Sixty-seven and 73 per cent respectively of the baby and adult sera had HI antibodies, the median titres being 2.0 and 2.8 $\log_{10}$ units per ml respectively (Tables 186 and 187). However, all the young adults between the ages of 18 and 29 years had HI antibodies in their sera whereas only 3 of the 7 adults over 30 years of age had antibodies. The median values for young and old adults were respectively 2.5 and 1.3 $\log_{10}$ units per ml.

Forty and 67 per cent respectively of the baby and adult sera had CF antibodies, the median titres being 1.7 and 2.0 $\log_{10}$ units per ml respectively (Tables 186 and 187). All but one of the young adults had CF antibodies in their sera whereas only 3 of the 7 adults over 30 years of age had antibodies. The median velues were respectively 1.8 and 1.6.

Neutralising antibody titres of baby sera ranged from 2.42 to 3.15 $\log_{10}$ units per ml and of adult sera ranged from 2.64 to 3.85 $\log_{10}$ units per ml. HI antibody titres were lower and CF antibody titres were the lowest. There were, however, direct relationships

which were linear and significant between the three types of antibodies (Table 188; Fig. 69). The relationships of one type of antibody to the other two types were similar although the antibody levels differed significantly (Table 189).

MONKEYS

As with human convalescent sera the N titres of simian sera were higher than the HI titres which in turn were significantly higher than the CF titres ($t = 2.277^{**}$; $P < 0.025$ (Table 190).

RABBITS

Attempted Experimental Infection: Inoculation of rabbits with live measles virus produced no clinical syndromes and there was no evidence of viral replication in the blood, spleen or lymph nodes. When heparinised blood and suspensions of spleen and lymph nodes that were harvested at intervals ranging from 6 hours to 7 days post-inoculation were seeded on to LLC-MK2 or FL cells no CPE occurred up to 21 days. Likewise, cultures prepared from blood leucocytes, spleen and lymph node cells from the inoculated rabbits developed no CPE in an observation period of 14 to 20 days.

Attempts to establish measles virus in rabbits by serial passage of blood from the inoculated rabbits to healthy rabbits failed. The procedure employed was to

inoculate a pair of healthy rabbits intravenously with 5 ml of pooled heparinised blood from three rabbits that were inoculated three days previously with 5 ml of $10^{5.6}$ TCD_{50} per ml of virus intravenously. Blood collected on the 4th day from the 1st passage rabbits was inoculated into two healthy rabbits; in all, seven serial passages were carried out. When the rabbits of the different passage levels were inoculated on the 20th day with 2 ml of Behringwerke Tween-ether haemagglutinins having an HA titre of 3.4 $\log_{10}$ units per ml, the resulting HI antibody response was primary in nature (Table 191). The median HI antibody response in the rabbits was similar to that of a 2nd group of rabbits which were first inoculated with normal rabbit blood and 20 days later with Tween-ether haemagglutinins (Table 192). On the other hand, rabbits that were sensitized with measles virus responded anamnestically to reinoculation with haemagglutinins (Table 193).

HI Antibodies:

Response to one dose of live virus: Rabbits inoculated intravenously with measles virus developed HI antibodies, the degree of response being related to the amount of virus inoculated such that higher doses always induced antibodies whereas low doses did not (Table 194). Moreover, thetitres of antibodies at three weeks were directly related to the titres of virus inoculated (Fig. 70).

To study the onset of HI antibodies eight rabbits were inoculated intravenously with $10^{5.6}$ TCD_{50} per ml of virus. Seroconverstion occurred in seven of the rabbits on the 7th day. At three weeks all rabbits had HI antibodies (Table 195). Maximum titres were reached at four weeks; however, individual variations existed. The titres persisted up to ten months, the longest observation period (Table 196).

Response to heated haemagglutinins: Eight rabbits were inoculated intravenously with 2 ml of Wellcome heat-inactivated haemagglutinins having an HA titre of 2.5 $\log_{10}$ units per ml. Both in the onset of HI antibodies and in the rate of seroconversion the response of the rabbits was similar to the response of rabbits inoculated with live virus (Table 197). Similarly, the antibodies persisted for at least ten months (Table 196).

Response to Tween-ether haemagglutinins: Thirty-three rabbits received intravenously 2 ml of Behringwerke Tween-ether haemagglutinins having an HA titre of 3.4 $\log_{10}$ units per ml (Table 198). As before, HI antibodies were detected in a high percentage on the 8th day and all rabbits had HI antibodies on the 12th day (Table 196).

Summary: The antibody responses of rabbits to single doses of live virus, heated haemagglutinins and Tween-ether haemagglutinins were similar (Fig. 71).

Response to two doses of the haemagglutinins: Two

rabbits, Nos. 24/67 and 25/67, were inoculated with 2 ml of Wellcome heat-inactivated haemagglutinins having a titre of 2.2 $\log_{10}$ units per ml; 0.5 ml of the inoculum was given intravenously and the rest intramuscularly at multiple sites after emulsifying with equal volume of Freunds' incomplete adjuvant. On the 7th day a 2nd inoculation was given similarly (Table 199). A 2nd group of rabbits, Nos. 26/67 and 27/67, was inoculated likewise with two doses of 2 ml on each occasion with Behringwerke haemagglutinins (3.4 $\log_{10}$ units per ml) (Table 199). The median titres of HI antibodies in the two groups of rabbits were far higher than the titres in rabbits that received one inoculation of the two haemagglutinins (Fig. 72). Comparison of the combined and individual responses between the two groups of rabbits inoculated with two doses of the Wellcome and Behringwerke haemagglutinins revealed similarities in both the onset and intensity of HI antibody responses ($F = 0.52$; d.f. 1, 12). High levels of antibodies persisted for at least ten months (Table 200).

Anamnestic responses: The effect of reinoculation of live virus or haemagglutinins on the secondary HI antibody response of rabbits sensitized with live virus or haemagglutinins was investigated. It was found that irrespective of the primary inoculum rabbits re-inoculated weeks or even months later with live virus or Tween-ether haemagglutinins manifested a dramatic

anamnestic response (Tables 201-204; Fig. 73). In some groups the anamnestic response was detected as early as two days after secondary inoculation. The intensity of the anamnestic response was not influenced by the levels of antibodies that existed at the time of reinoculation ($F = 2.66$; d.f. 1, 12).

Haemadsorption-Inhibition Antibodies: The time of induction and development of titres of haemadsorption-inhibition (HAdI) antibodies were similar to the onset and development of the HI antibodies irrespective of whether the rabbits received live virus or haem-agglutinins but the HAdI titres were lower than the HI titres (Tables 205 and 206; Fig. 74).

CF Antibodies: Non-specific fixation of measles CF antigens and of negative control antigens by normal rabbit sera was encountered in about 40 per cent of sera but the titres rarely exceeded 1.9 $\log_{10}$ units per ml (Table 207). Sometimes the non-specific fixation was thermolabile at 60°C for 15-20 minutes and was removed by treatment with kaolin or acetone.

In contradistinction to the early onset of HI antibodies, the CF antibodies appeared at two weeks in most rabbits receiving one dose of live virus or haem-agglutinins (Tables 207 and 208; Fig. 74). Furthermore, the CF antibody titres were about 0.2 to 0.6 $\log_{10}$ units lower than the HI titres. The antibodies persisted for 8-12 weeks.

Immuno-Diffusion Antibodies: Following intravenous inoculation of one dose of live virus, heated haem-agglutinins or Tween-ether haemagglutinins, immuno-diffusion (ID) antibodies were demonstrable in 40, 0 and 12 per cent of rabbits respectively at two weeks post-inoculation (Table 209). With two doses, all the inoculated rabbits developed ID antibodies one week after the 2nd inoculation (Table 209).

Sera of rabbits inoculated with two doses of Wellcome heat-inactivated or Behringwerke Tween-ether haemagglutinins generally produced two precipitation bands, or occasionally, a third when diffused against cell culture virus or Behringwerke haemagglutinins (Table 209; Fig. 75). One of 11 rabbits inoculated with two doses of Behringwerke haemagglutinins yielded serum that occasionally produced a 4th band when diffused against virus or Behringwerke haemagglutinins. Likewise, an occasional 4th band appeared in agar gels when serum from a rabbit that was given heated haem-agglutinins first and cell culture virus later was diffused against the virus (Fig. 75).

Sera of rabbits inoculated with one dose of live virus generally produced two diffusion bands when allowed to react with sonicated measles-infected cells; occasionally three diffusion bands formed (Fig. 76). The precipitation bands were virus-specific as sera of rabbits inoculated with the virus propagated in Hep-2

or FL cells reacted with measles-infected LLC-MK2, PMK or PCK cells producing two or three precipitation lines. Furthermore, no reaction occurred when the sera were diffused against uninfected Hep-2 or FL cells, or concentrated, uninfected cell culture fluid.

Sera of rabbits inoculated with two doses of virus or one dose of virus followed by one dose of heated or Tween-ether haemagglutinins contained ID antibodies which produced three to five precipitation bands when diffused against cell cultured virus. Two of the bands appeared to be non-viral since adsorption of sera with concentrated, freeze-dried, uninfected cell culture fluids removed them. In some trials the 4th band failed to develop after treatment of the serum with sonicated uninfected human liver cells or liver powder but in other trials it developed. The reactions were inhibited by prior incubation of the sera with measles cell culture ID antigens (Fig. 76).

Neutralization Antibodies: The titres of N antibodies in rabbits inoculated with a single dose of live virus or Tween-ether haemagglutinins ran parallel to the titres of the HI, HAdI, CF and ID antibodies; but the N antibody titres were always the highest (Tables 210 and 211).

CATTLE

Attempted Experimental Infection: Young calves

inoculated subcutaneously or intravenously with measles virus developed neither clinical syndromes nor viraemias. No CPE developed in LLC-MK2 cells inoculated with blood collected from the calves at different post-inoculation periods. Leucocyte cultures prepared from the blood samples likewise failed to show CPE.

HI Antibodies:

Response to live virus: Calves inoculated with a single dose of live measles virus developed HI antibodies (Tables 212 and 213). The degree of response appeared to be related to the amount of virus inoculated such that the higher dose always induced higher levels of antibodies (Fig. 77). However the difference between the titres was not statistically significant ($t = 0.611$, $P > 0.50$). Seroconversion was apparently dose-dependent; seroconversion in four calves inoculated subcutaneously with $10^{5.5}TCD_{50}$ of live measles virus took 28 days whereas it was only seven days in four calves inoculated with $10^{5.9}TCD_{50}$ of live measles virus.

The sera of three calves contained HI antibodies six months but not eight months after inoculation. The sera of the other calves still had antibodies nine months after inoculation and two calves at least had antibodies at 11.5 months (Table 214; Fig. 78).

Response to haemagglutinins: Four calves were injected subcutaneously with 10 ml of heated Wellcome

haemagglutinins having a titre of $10^{2.8}$ units per ml. One calf failed to respond. The other three calves developed low levels of HI antibodies three weeks later and the antibodies persisted unchanged in two calves for at least eight weeks. No antibodies were detected in the third calf after the sixth week (Table 215; Fig. 78).

Anamnestic responses: As with rabbits, calves sensitized by a single dose of live measles virus or haemagglutinins responded anamnestically when re-inoculated with live virus or haemagglutinins (Fig. 79). The HI antibody titres after challenge in three of the four groups of calves were significantly higher than the titres before challenge (Tables 216 and 217) and, similarly, the titres of the secondary response measured two weeks after challenge were all significantly higher than the titres two weeks after the primary exposure (Tables 218 and 219).

Colostral antibodies: Three pregnant cows were twice injected subcutaneously with $10^{6.2}TCD_{50}$ of measles virus, the second dose being given seven days after the first. HI antibodies were detected in the sera of the cows seven days after the first dose and at the time of parturition had reached substantial levels (Table 220). In contrast, a 4th cow that was twice injected with $10^{4.4}$ units of Tween-ether haemagglutinins developed low levels of HI antibodies (Table 221).

The colostrums of all four cows contained HI anti-

bodies and the antibody titres four to eight hours after calving were higher than the corresponding serum titres (Table 222). The decline in colostrum antibody was rapid, linear and significant in all but the colostrum from the cow injected with Tween-ether haemagglutinins, the half-life periods varying from 7.9 to 9.1 hours whereas the titres of serum antibody remained virtually constant (Table 223). The differences in the slopes of the regressions of the serum and colostrum antibody titres were significant except in the cow injected with Tween-ether haemagglutinins (Table 224; Fig. 80). There were, however, no significant differences between the regression slopes of colostrum antibody in the four cows although the levels differed significantly ($F = 0.06$ and $F = 6.79^{**}$ respectively, degrees of freedom 3, 12).

Colostrally-derived antibodies: At birth the sera of the calves born of the cows injected with live measles virus or haemagglutinins did not have HI antibodies. The antibodies were, however, detectable in the calves' sera six to eight hours after the calves had ingested colostrum and the antibody titres increased to maxima 12 to 18 hours after sucking (Table 225). The rises in the antibody titres approached linearity (Table 226) and collectively were highly significant ($F = 62.68^{**}$; d.f. 1, 11) with similar slopes ($F = 1.76$; d.f. 3, 11). The maximal titres were directly related

to the first observed titres ($F = 34.91^{**}$; d.f. 3, 11).

Administration of live measles virus, measles haemagglutinins or live distemper virus to calves possessing colostrally-derived HI antibodies affected neither the antibody titres nor the rates of their decline (Table 227). The declines in the antibody titres approached linearity and collectively were highly significant ($F = 34.42^{**}$; d.f. 1, 18) with similar slopes ($F = 1.15$; d.f. 3, 18) (Table 228). The mean half-life period of the colostrally-derived HI antibody calculated from the significant regressions was 15.71 days (Table 228).

Neutralising Antibodies: One and two infections of live measles virus induced the appearance of neutralising antibodies in the sera of cattle (Table 229). The titres of neutralising antibodies were marginally higher than the titres of HI antibodies.

Immuno-diffusion Antibodies: No immuno-diffusion antibodies were detected in the sera of calves or cows injected with one or two doses of live measles virus or haemagglutinins.

SHEEP

Attempted Experimental Infection: Sheep, like rabbits and calves, were clinically refractory to inoculation with live measles virus. Attempts to recover virus from the bloods of inoculated sheep failed.

HI Antibodies: HI antibodies were detected in the sera of three out of eight sheep inoculated three to four weeks earlier with $10^{4.5}TCD_{50}$ of measles virus (Table 230). On the other hand all three sheep inoculated with $10^{5.2}TCD_{50}$ of measles virus developed HI antibodies which appeared earlier than those induced by the lower dose of virus (Table 231).

Neutralising Antibodies: Sheep, like rabbits and calves, developed neutralising antibodies after a single dose of live measles virus. The titres of the neutralising antibodies were like wise marginally higher than the titres of HI antibodies (Table 232).

Immuno-diffusion Antibodies: These were not detected in the sera of sheep inoculated with one or two doses of live measles virus. However, a sample of sheep anti-measles hyperimmune serum received from the Medical Research Council Laboratories, Holly Hill, contained 1.3 $\log_{10}$ units per ml of ID antibodies. The titres of HI, CF and N antibodies are respectively 3.7, 3.1 and 4.12 $\log_{10}$ units per ml.

GUINEA PIGS

Two guinea pigs were inoculated intraperitoneally with 1 ml of $10^{6.2}TCD_{50}$ of live measles virus and two guinea pigs were similarly inoculated with the same amount of the virus exposed to 60°C for 30 minutes. The preparation had a titre of 1.8 $\log_{10}$ units per ml of

haemagglutinins. Haemagglutination-inhibition, complement fixing and neutralising antibodies appeared in the sera of both groups of guinea pigs (Table 233). Differences in antibody titres were not attributable to the inocula ($F = 2.29$; d.f. 1, 6) but there were differences in titres between the types of antibodies ($F = 6.93^{*}$; d.f. 4, 6); neutralising antibodies attained titres higher than those of complement fixing antibodies ($t = 5.588^{**}$; P 0.01) and haemagglutination-inhibition antibodies ($t = 2.604^{*}$; P 0.05). Differences between the titres of complement fixing and haemagglutination-inhibition antibodies were not significant ($t = 1.974$; P 0.05).

DISTEMPER ANTIBODIES

DOGS

Sera from 20 out of 34 pups, 2 to 22 days old and belonging to 9 litters, had distemper CF antibodies, the titres of which varied from 1.6 to 2.5 (Table 234). There was a significant, inverse, linear relationship between the age of the pups and the CF antibody titres ($F = 8.07^{**}$; d.f. 1, 20).

Sera from 84 dogs of different ages and backgrounds were also examined for distemper CF antibodies. Of the 14 alleged clinical cases of canine distemper of 6 to 14 days duration, 8 had low titres of CF antibodies (Table 235). Sixteen out of 20 dogs that had manifested "clinical distemper" for more than 14 days had significantly higher levels of antibodies ($t = 4.235^{**}$; $P < 0.01$).

Distemper CF antibodies were detected also in 53 per cent of dogs with a history of past distemper, in 61 per cent of dogs with a history of distemper vaccination and in 59 per cent of dogs with an unknown history (Table 236). No relationship, however, was detected between the ages of these dogs and the antibody titres ($F = 0.05$; d.f. 1, 14).

In dogs with a distemper history a close correlation existed between the CF and N antibody titres ($F = 30.06^{**}$; d.f. 1, 8) (Table 237; Fig. 81). The N antibody titres were, however, significantly higher

than the CF titres ($t = 2.260^{**}$; $P < 0.01$). In none of the sera distemper immuno-diffusion antibodies was detected.

RABBITS

Attempted Experimental Infection: No clinical syndromes developed in 8 rabbits following intravenous inoculation with $10^{4.8}TCD_{50}$ of the Rockborn strain of canine distemper virus. Leucocyte cultures prepared from heparinised blood collected from the rabbits at 6, 12, 24, 72 and 96 hours post-inoculation manifested no CPE in 18 days. Likewise, the blood collected at these intervals and seeded into monolayers of MDCK cells failed to induce detectable CPE in 25 days. Two further blind passages failed to reveal evidence of virus.

CF Antibodies:

Response to live virus: Thirteen rabbits were inoculated intravenously with 1 ml of $10^{4.5}TCD_{50}$ of the Rockborn strain of canine distemper virus. Simultaneously a similar dose of the virus was inoculated intramuscularly into multiple sited in 0.25 ml amounts after emulsifying with 1 ml of Freunds' incomplete adjuvant. Antibodies reactive to distemper EF antigens were detected in four rabbits on the 7th day post-inoculation (Tables 238-240). Seroconversion was 100 per cent at two weeks. Maximum titres were reached

at four weeks.

Effect of the number of doses: To study the influence of the number of doses of the virus on the onset and intensity of the CF antibody response, rabbits were re-inoculated with the same amounts of the virus intravenously and intramuscularly at weekly intervals. Individual titres were higher in rabbits injected with two and three doses of the virus than in those injected with one dose only (Tables 238-240; Fig. 82). The median titres at two weeks in the three groups were 1-9, 2-80 and 3-25 respectively. Furthermore, there was a significant, positive, linear relationship between the number of doses inoculated and the mean antibody titres ($F = 35.32^{**}$; d.f. 1, 11) (Fig. 83).

Response to heat-inactivated virus: Three rabbits were inoculated intravenously with 1 ml of $10^{4.5}$ TCD_{50} of the Rockborn strain of canine distemper virus that was exposed to 60°C for 30 minutes. Simultaneously a similar amount of the inactivated virus was inoculated intramuscularly at multiple sites after emulsifying with 1 ml of Freunds' incomplete adjuvant. The inoculations were repeated on the 7th day. The CF antibody titres were similar to the titres in rabbits inoculated with one dose of live virus in both the time of onset and value ($t = 0.126$; $P > 0.5$) (Table 241), but were significantly lower than the titres developed in rabbits inoculated with two doses of live virus ($t = 3.406^{**}$;

$P < 0.01$).

Immuno-diffusion Antibodies: No ID antibodies were detected in rabbits injected with one dose of live virus or two doses of heat-inactivated virus (Table 242). On the other hand, in five out of eight rabbits inoculated with two doses of live virus ID antibodies were detected on the 7th day after the second inoculation. Seroconversion was 100 per cent at four weeks. The onset and rate of seroconversion was apparently faster in rabbits inoculated with three doses of live virus. However, the relationship between the number of doses inoculated and the intensity of ID antibody response was not statistically significant ($F = 6.13$; d.f. 1, 4) (Tables 243 and 244).

Neutralising Antibodies: Rabbits inoculated with live distemper virus developed neutralising antibodies (Tables 245 and 246). The intensity of the response was apparently related to the number of doses administered but the data were not statistically significant ($t = 1.006$; P 0.4). The neutralising antibody titres in rabbits inoculated with two doses of inactivated distemper virus were marginal and did not differ significantly from the titres induced by a single dose of live virus ($t = 1.517$; P 0.3) (Table 247).

Summary: Rabbits inoculated with the Rockborn strain of distemper virus developed CF, ID and N antibodies, the titres being related to the number of doses (Fig. 84).

The titres induced by the live virus were significantly higher than those induced by the inactivated virus. The N antibody titres were always higher than the CF titres. However, the titres of ID antibodies were poor.

CALVES

Attempted Experimental Infection: Calves were found refractory to canine distemper virus. There was no clinical and virological evidence of infection in three calves inoculated subcutaneously with $10^{5.5}TCD_{50}$ of the Rockborn strain of canine distemper virus.

CF Antibodies: As with rabbits, development of CF antibodies in calves was positively and significantly related to the number of doses of the live virus inoculated ($F = 12.5^{*}g$; d.f. 1, 5) (Tables 248 and 249). On the other hand, the antibody response of calves inoculated with two doses of $10^{5.5}TCD_{50}$ of distemper virus that had been inactivated at $60^{o}C$ for 30 minutes was poor in that only two out of five calves developed low titres of antibodies (Table 250).

ID Antibodies: Immuno-diffusion antibodies were not detected in the sera of calves irrespective of the number of doses of live or heat-inactivated virus inoculated.

CROSS-RELATIONSHIPS

MEASLES AND DISTEMPER

Anti-Distemper Sera:

Measles haemagglutination-inhibition: Fifteen out of 139 canine sera had antibodies reactive to measles Behringwerke Tween-ether haemagglutinins (Tables 251 and 252). Of these, 17 and 25 per cent respectively were from cases of "clinical distemper" of more than 14 days' duration and from dogs that had a distemper "history". There was no relationship between the age of the dogs and the HI antibody levels ($F = 0.934$; d.f. 1, 15).

None of the sera of rabbits and calves inoculated with single or multiple doses of live or heat-inactivated Rockborn strain of canine distemper virus propagated in MDCK cells contained antibodies to measles Tween-ether haemagglutinins (Table 253). The sera, however, had different levels of distemper CF antibodies four weeks after inoculation. Similarly, rabbits inoculated at weekly intervals with three doses of the CTVM and the Onderstepoort strains of canine distemper virus failed to develop antibodies reactive to measles haemagglutinins, although they developed antibodies to distemper CF antigens (Table 254).

Measles haemadsorption-inhibition: Of the eight canine sera that inhibited measles haemagglutination, only one prevented haemadsorption of baboon erythrocytes by measles-infected LLC-MK2 monolayers; however, the

titre was poor (Table 255). Likewise, sera of rabbits hyperimmunised with distemper virus strains contained no antibodies that inhibited measles haemadsorption.

Measles complement-fixation:

Canine sera: Most canine sera with moderate titres of antibodies reactive to measles haemagglutinins also reacted specifically with measles CF antigens (Table 256). The titres of the two types of antibodies were of the same order. On the other hand, the titres of the antibodies reactive against measles CF antigens were collectively and individually lower than the titres of antibodies to distemper CF antigens, although the differences were not statistically significant ($t = 1.731$; $P > 0.20$). There were marked differences, however, between the titres of homotypic and heterotypic maternally-derived CF antibodies in the sera of 12 pups (Table 257). None of the pup sera contained antibodies reactive to measles haemagglutinins.

Rabbit sera: Sera of rabbits sensitized intravenously with one dose of live distemper virus contained no antibodies reactive to measles CF antigens. However, the titres of distemper CF antibodies were low to moderate (Table 258). Likewise, only one out of three rabbits inoculated intravenously at weekly intervals with two doses of heat-inactivated Rockborn strain of canine distemper virus showed antibodies to measles CF antigens, but, the titre was poor. On the

other hand, all rabbits inoculated with two or three doses of live distemper virus produced antibodies to measles CF antigens (Tables 258 and 259). There was no relationship between the number of doses inoculated and the titres ($t = 0.009$; $P > 0.10$).

Measles immuno-diffusion:

Direct tests: Sera of rabbits inoculated with different strains of distemper virus did not produce precipitation lines in agar-gel when diffused against concentrated measles cell culture virus or haem-agglutinins (Table 260). The reaction was negative even when the sera were concentrated approximately eight times by forced dialysis against polyethylene glycol and then diffused against measles antigens.

Adsorption tests: Rabbit and dog anti-distemper sera having high titres of homologous ID antibodies were incubated in 0.5 ml amounts with different concentrations of measles cell cultures ID antigens or Behringwerke Tween-ether haemagglutinins at $4^{o}C$ for about 24 hours. The mixtures were then spun at 10,000 r.p.m. for 45 minutes and the supernatant fluids titrated for distemper ID antibodies. There was little difference between the titres before and after adsorption irrespective of the type of antigens used (Table 261). In control tests in which the sere were adsorbed with distemper ID tissue antigens, marked reduction of the specific antibody titres occurred

(Table 262); the decline in titre was significantly related to the dose of the antigens used (F = 8.97*; d.f. 1, 4) and was similar in rabbit and canine sera although the levels differed significantly (F = 0.52 and F = 11.93*; d.f. 1, 4) (Fig. 85).

In another experiment, distemper ID tissue antigens were treated with distemper and measles hyperimmune sera at 4°C for 18-20 hours. Adsorption of the antigens occurred with rabbit and dog anti-distemper sera but not with rabbit anti-measles sera (Table 263).

Measles neutralization: The sera of dogs with a distemper history that had measles CF antibodies also contained neutralising antibodies to measles and distemper viruses. The homologous antibody titres were higher than the heterologous antibody titres, although the values were not statistically significant ($t = 1.731$; $P > 0.20$) (Table 256).

Similarly, rabbits inoculated with two doses of live distemper virus developed antibodies which neutralized both viruses. The titres of antibodies reactive to distemper virus four weeks after inoculation were significantly higher than the titres of antibodies neutralising measles virus ($t = 5.268^{**}$; $P < 0.01$) (Table 264).

Anti-Measles Sera:

Distemper complement-fixation:

Human sera: Sera of nine babies were tested for

antibodies reactive to measles and distemper CF antigens. None had antibodies to distemper CF antigens, but six had homologous CF antibodies (Table 265). In contrast, 60 per cent of adult human sera tested had antibodies to distemper antigens. No relationship was demonstrable between the age of the subjects and the antibody titres ($F = 0.79$; d.f. 1, 4).

Simian sera: Most simian sera contained antibodies to distemper CF antigens. There was a close correlation between the titres of the homologous and heterologous CF antibodies, but the titres of the former were significantly higher ($t = 4.786^{**}$; $P < 0.01$) (Table 266).

Rabbit sera: All rabbits inoculated with one dose of either live measles virus or heated haemagglutinins developed antibodies that fixed complement in the presence of distemper CF antigens (Table 267). Similarly, most rabbits sensitized with one dose of Behringwerke Tween-ether haemagglutinins developed antibodies to distemper CF antigens. No differences existed between the titres of the antibodies induced by live virus or heated haemagglutinins or Tween-ether haemagglutinins ($F = 2.54$; d.f. 2, 14). In rabbits inoculated with heated haemagglutinins no correlation was demonstrable between the number of doses inoculated and the distemper CF antibody titres. The titres induced by two doses of the product were similar to the titres induced by one dose ($t = 0.875$; $P > 0.3$).

However, the antibodies persisted longer in rabbits inoculated with two doses.

Distemper immuno-diffusion:

Direct tests: Measles convalescent human and simian sera did not react with measles or canine distemper ID antigens in agar gel (Table 268). Similarly, calves inoculated with two doses of live measles virus or haemagglutinins developed no antibodies reactive to the homologous and heterologous ID antigens. A commercially prepared sheep anti-measles serum, however, did react with measles virus and haemagglutinins

Anti-measles sera prepared in rabbits by injecting intravenously multiple doses of live virus or haem-agglutinins contained antibodies that formed precipitation lines with measles virus or haemagglutinins in agar gel; the sera failed to react with distemper tissue or cell culture ID antigens (Table 268).

Adsorption tests: Titres of measles ID antibodies in rabbit anti-measles sera were either unaffected or slightly reduced when adsorbed for about 24 hours at $4^{\circ}C$ with distemper ID antigens (Table 269). Similar treatment with measles cell culture ID antigens or haem-agglutinins resulted in partial or complete adsorption of the specific ID antibodies (Table 270). The rate of adsorption of the homologous antibodies was positive and linear and significantly related to the titres of the two types of antigens ($F = 45.74^{**}$; d.f. 1, 4 and

F = 22.57; d.f. 1, 5 respectively). The two regression slopes were similar (F = 2.26; d.f. 5, 9) but the levels differed significantly (F = 4.33**; d.f. 5, 9) (Fig. 86).

Measles immuno-diffusion cell culture antigens and Tween-ether haemagglutinins were largely unaffected following adsorption at 4°C for 24 hours with dog and rabbit anti-distemper sera (Table 271; Fig. 97). However, the titres of the antigens were depressed when treated similarly with rabbit anti-measles sera.

Distemper neutralization: Four out of nine specimens of human measles convalescent sera contained low or marginal titres of neutralising antibodies to canine distemper virus (Table 272). As with measles CF antibodies, the titres of measles N antibodies were higher than the titres of antibodies neutralising distemper virus ($t = 3.781^{**}$; $P < 0.01$).

A similar relationship existed between the two types of antibodies in the sera of rabbits, cattle and sheep inoculated with one or two doses of live measles virus (Table 273). When measles neutralising antibody titres were low, distemper neutralising antibodies were not detected.

Heterotypic Challenge:

Response to live distemper virus: Six rabbits that were sensitized with a single dose of live measles virus were reinoculated intravenously after an interval of

eight to ten months with 1 ml of $10^{4.8}TCD_{50}$ of the Rockborn strain of canine distemper virus. At the time of reinoculation the median HI antibody titres of the rabbits was 2.35 (Table 274). At four days after reinoculation there was an anamnestic response in three out of four rabbits characterized by the development of increased titres of measles HI and measles CF antibodies (Tables 274 and 275; Fig. 87). There was also a concomitant increase in the levels of distemper CF antibodies, the titres being higher than those previously encountered in the primary response of rabbits exposed to live distemper virus. At eight days the levels of measles HI and measles CF antibodies were significantly higher than the levels at the day of reinoculation ($t = 3.768^{**}$; $P < 0.01$ and $t = 3.989^{**}$; $P < 0.01$ respectively). However, there was no significant difference in the levels of the two types of antibodies ($t = 1.043$; $P > 0.2$). The antibodies to distemper CF antigens also rose markedly.

A similar pattern of heterotypic anaemnestic responses ensued in a second group of six rabbits first inoculated with measles haemagglutinins and reinoculated 6-8 months later with live distemper virus (Tables 276 and 277; Fig. 87). The titres of measles HI and measles CF antibodies were significantly higher at eight days ($t = 2.482^{*}$; $P < 0.05$ and $t = 4.749^{**}$; $P < 0.01$ respectively).

Cattle sensitized subcutaneously with live measles virus or Tween-ether haemagglutinins and challenged subcutaneously 12 weeks later with $10^{5.3-5.8}TCD_{50}$ of canine distemper virus likewise developed anamnestic measles HI antibodies (Tables 278 and 279; Fig. 88).

The anamnestic increases in HI antibody titres were linear and significant in both rabbits and calves (Fig. 89). The rates of increase were similar although the levels differed significantly ($F = 0.34$ and $F = 27.71^{**}$; d.f. 3, 72).

Response to inactivated distemper virus: Rabbits were sensitized intravenously with live measles virus or haemagglutinins and reinoculated 6-8 months later intravenously with $10^{4.9}TCD_{50}$ of the Rockborn strain of canine distemper virus that was exposed to 60°C for 30 minutes. There were no anamnestic measles antibody responses (Tables 280-283; Fig. 87).

Similarly, calves first inoculated subcutaneously with live measles virus or haemagglutinins did not show secondary measles antibody responses when challenged subcutaneously 3-4 months later with heat-inactivated distemper virus (Tables 284-286; Fig. 88). There was a marginal rise of distemper CF antibody, apparently a primary response. Even at 14 days after reinoculation the titres were poor.

Response to live measles virus: Three groups of rabbits were first inoculated intravenously with one,

two and three doses respectively of live, Rockborn strain of canine distemper virus. Each dose contained $10^{4.9}TCD_{50}$ per ml of the virus and the second and third inoculations were spaced at weekly intervals. Two months later the rabbits were reinoculated intravenously with 1 ml of $10^{5.3}TCD_{50}$ of the Holly Hill strain of measles virus. There was a quick anamnestic response characterised by the production of antibodies reactive to measles haemagglutinins and measles CF antigens (Tables 287 and 288). There was no significant correlation between the number of doses of the primary inoculum and the strength of the secondary HI antibody response ($F = 2.26$; d.f. 1, 12) but the development of the titres was faster in rabbits receiving three doses and two doses than in those injected with one dose only.

Exposure of the sensitized rabbits to live measles virus also resulted in a marked increase in the titres of distemper CF antibodies, although the difference was not statistically significant ($t = 1.525$; $P > 0.1$) (Table 288). Comparison of the development of the titres of antibodies reactive to measles and distemper antigens showed that the levels of the measles HI and measles CF antibodies were similar ($t = 0.752$; $P > 0.40$) but those of distemper CF antibodies were significantly different ($t = 2.964^{*}$; $P < 0.02$).

Similarly, calves first exposed to live distemper virus developed a rapid anamnestic measles HI antibody

response when challenged with live measles virus (Table 289).

Rabbits and calves first inoculated with one or more doses of heat-inactivated distemper virus did not respond anamnestically to reinoculation with live measles virus (Tables 290-292). There was a slow onset of measles HI antibodies, reminiscent of primary response to measles virus. The median titres at ten days were 1.6 and 1.3 respectively in rabbits and calves. However, sensitized rabbits developed measles CF antibodies anamnestically on reinoculation with live measles virus (Table 291). The antibodies were detected at four days in the three inoculated rabbits and the titres rose markedly at ten days. There was a concomitant distemper CF antibody response, the titres at ten days being significantly higher than the titres on the day of exposure to measles virus ($t = 2.828^{*}$; $P < 0.05$).

Response to measles haemagglutinins: Rabbits sensitized with two doses of live distemper virus responded anamnestically when reinoculated with Behringwerke Tween-ether haemagglutinins; the antibodies were reactive to the haemagglutinins (Table 293). Moreover, the response was of the same order as that developed in rabbits challenged with live measles virus following sensitization with live distemper virus ($F = 0.11$ and $F = 0.09$ respectively; d.f. 1, 31). There was also a significant increase in the titres of

antibodies reactive to measles CF antigens ($F = 10.74^{*}$; d.f. 1, 13) (Table 294). The anamnestic increases in CF and HI antibodies occurred at similar rates ($F = 3.90$; d.f. 1, 26), although the levels differed significantly ($F = 33.91^{**}$; d.f. 1, 26).

Reinoculation of calves with Tween-ether haemagglutinins 12 weeks after sensitization with live distemper virus resulted in significant anamnestic measles HI antibody levels ($F = 57.99^{*}$; d.f. 1, 7) (Table 295). The response was, however, significantly different in rates and levels from that of calves which were sensitized with live distemper virus and challenged later with live measles virus ($F = 2.71$ and $F = 9.14$ respectively; d.f. 1, 14), (Table 296).

Comparison of the anamnestic HI antibody titres in the sera of rabbits and calves first inoculated with live distemper virus and challenged with Tween-ether haemagglutinins revealed that they developed at similar rates and times ($F = 0.78$ and $F = 0.07$ respectively; d.f. 1, 49) (Fig. 90).

Maternally-Derived Antibodies: The responses to measles Tween-ether haemagglutinins and live distemper virus were investigated in a litter of seven pups, the offspring of a Harrier hound bitch that was inoculated subcutaneously twice at weekly intervals with $10^{3.8}TCD_{50}$ of "Caninovac",[1] 13 days before whelping. Two days

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1. Hoechst Pharmaceuticals Ltd., Brentford, Middlesex.

before parturition the distemper CF and neutralising antibody titres were 2.5 and 2.92 respectively. There were no detectable titres of measles HI, CF and N antibodies.

Three weeks after birth the pups were bled for sera and then inoculated in two groups, three males and four females, respectively with $10^{3.7}\log_{10}$ units of Behringwerke Tween-ether haemagglutinins subcutaneously and $10^{4.1}TCD_{50}$ of "Caninovac" intramuscularly; the distemper vaccine was emulsified with equal volumes of Freunds' incomplete adjuvant before inoculation. Four out of seven pups had low levels of maternally-derived distemper CF and N antibodies (Tables 297 and 298) but none had antibodies reactive to measles antigens (Tables 299 and 300).

At eight weeks the pups were bled and then re-inoculated with measles haemagglutinins and "Caninovac", those first inoculated with the haemagglutinins receiving the distemper vaccine and vice versa. As before, the distemper vaccine was emulsified with Freunds' incomplete adjuvant and administered intramuscularly.

Primary homotypic responses: Following inoculation with measles haemagglutinins the three pups developed poor titres of measles HI, CF and N antibodies (Table 299). The response of pup 1 was not impaired by the presence of maternally-derived distemper antibodies.

The pups (Nos. 4-7) inoculated with live distemper vaccine developed moderate to good titres of distemper CF and N antibodies (Tables 298). The presence of maternally-derived distemper antibodies in pups 4, 6 and 7 had not apparently interfered with their responses to distemper virus.

Primary heterotypic responses: The pups sensitized with measles haemagglutinins also developed antibodies reactive to distemper virus and distemper CF antigens, but the titres were barely detectable (Table 297). Likewise, distemper virus stimulated the production of low titres of antibodies to measles antigens (Table 300). However, in one pup, neutralising antibodies to measles virus were not detected.

Secondary responses: Pups sensitized with measles haemagglutinins developed anamnestic measles N antibodies to significantly high levels on reinoculation with distemper virus (Tables 299 and 301; Fig. 91). There was a rise in the CF titres, although not statistically significant. The HI titres, on the other hand, were depressed significantly. The anamnestic response was also manifested by the distemper CF and N antibodies, the rises being significant (Tables 297 and 301).

Pups exposed to live distemper virus responded anamnestically to reinoculation with measles haemagglutinins producing significantly high titres of

measles HI, CF and N antibodies (Tables 300 and 302; Fig. 92). Distemper CF and N antibody titres were not affected (Tables 298 and 302).

MEASLES AND RINDERPEST

Anti-Rinderpest Sera:

Measles haemagglutination-inhibition:

Bovine acute phase sera: Three out of eight Indian Hill Zebu cattle inoculated subcutaneously with the virulent bovine Hissar strain of rinderpest virus developed low titres of antibodies reactive to measles Tween-ether haemagglutinins on the 6th or 7th post-infection day (Table 303). No differences existed either in the onset or the titres of the antibodies in the sera of killed or dying animals (Table 304). Virus was recovered from all animals tested and specific ID antigens were also demonstrated in the lymph nodes.

Bovine convalescent phase sera: In two out of three cattle recovering from experimental rinderpest infection HI antibodies were detected on the 7th post-infection day. The levels increased steadily reaching a maximum at two or three weeks (Table 305).

Goat sera: Eight Nigerian Dwarf goats were inoculated subcutaneously with the caprine strain of rinderpest virus. Antibodies reactive to measles haemagglutinins were detectable in three goats between the 8th and 10th post-inoculation day (Table 306).

Seroconversion was complete in all but one at two weeks. Maximum antibody levels were generally reached at three weeks post-inoculation, after which the titres dropped or were maintained. Antibodies were not demonstrable in three out of four goats at seven weeks.

Pig sera: Twenty-one West African Dwarf pigs were injected subcutaneously with the caprine strain of rinderpest virus. Thirteen pigs manifested mild to moderate malaise, elevation of temperature, anorexia and leucopenia. Virus was recovered from the two cases tested. Cases were classified as abortive when the clinical symptoms were mild. Measles HI antibodies were detected in 9 out of the 13 pigs that manifested clinical symptoms and in all pigs that were clinically negative (Table 307). In the first group the titres were not related to the severity of the symptoms ($t = 0.155$; $p > 0.05$). There were, however, significant differences in the levels of the antibodies between the two groups of pigs such that those exhibiting overt signs had the lower titres ($t = 2.515^*$; $P < 0.025$).

Vaccinated cattle sera: Sera of 27 White Fulani dairy cows, aged $2\frac{1}{2}$ to 12 years and with a history of multiple vaccination with live, lapinized and cell culture-adapted bovine strains of rinderpest virus were examined for antibodies reactive to measles haem-agglutinins. Twenty-three (85 per cent) had antibodies

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435 days after the previous vaccination; the titres ranged from 1.3 to 2.8 (Table 308). There was no correlation between the number of vaccinations and the titres of the HI antibodies ($F = 0.24$; d.f. 1, 25).

The sera of four cows contained no HI antibodies and remained negative in three cows despite re-inoculation.

When the cows were challenged subcutaneously with cell culture-adapted bovine strain of rinderpest virus 19 responded, although only 6 anamnestically (Table 309; Fig. 93). Neither the percentage nor the degree of response was related to the pre-existing antibody levels or to the number of inoculations the animals had previously received. When tested after a further period of 402 days, the same 23 cows were found to have HI antibodies. As before, the titres of the persisting antibodies were not related to the vaccination history of the cows. Furthermore, comparison of the antibody levels at 22 and 402 days post-reinoculation revealed no significant differences ($t = 0.797$; $P > 0.05$).

Colostrally-derived antibodies: Twenty-seven calves of different ages, the progeny of the above 27 dairy cows, were examined for antibodies reactive to measles haemagglutinins. Eight had antibodies, the titres of which ranged from 1.3 to 2.5 (Table 310). The antibody levels were apparently related to age of the calves but the data were not statistically significant ($F = 4.35$; d.f. 1, 6).

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Nineteen calves, eight of which had measles HI antibodies and 11 without antibodies, were inoculated subcutaneously with cell culture-adapted rinderpest virus. There was no development of antibodies reactive to measles haemagglutinins in the first group of calves (Table 310; Fig. 94). In three calves a slight reduction in the antibody levels was evident at two weeks post-inoculation. A further slight decline in the titre occurred in another calf a week later. The half-life of the colostrally-derived antibodies was 33 days.

In the second group antibodies reactive to measles haemagglutinins developed in seven calves at two to three weeks post-inoculation (Table 311). There was no significant fall in titres in five calves in 365 days ($t = 1.414$; $P > 0.05$). Four calves aged 147, 203, 205 and 232 days respectively did not respond to the vaccination in an observation period of 22 days (Fig. 94).

In another trial, eight calves, aged from 164 to 298 days, and without measles HI antibodies, were inoculated subcutaneously with the caprine strain of rinderpest virus. Antibodies reactive to measles haemagglutinins developed in four out of the eight calves at one week post-inoculation. Significant elevation of titres occurred at two weeks ($F = 42.18^{**}$; d.f. 1, 14) (Table 312; Fig. 95).

Haemadsorption-inhibition: Anti-rinderpest sera with high titres of measles HI antibodies inhibited measles haemadsorption. Those with low titres did not (Table 313).

Measles complement-fixation: Some specimens of rabbit anti-rinderpest sera having good titres of antibodies reactive to measles haemagglutinins also reacted with measles CF antigens. Others did not (Table 314).

Neutralization of measles virus: Neutralising antibodies to the Holly Hill strain of measles virus were demonstrated in cattle, goat and rabbit anti-rinderpest sera. The N antibody titres were higher than the CF and HI antibody titres (Table 314).

Measles immuno-diffusion: Antibodies reactive to measles Tween-ether haemagglutinins and cell culture ID antigens were not demonstrable in anti-rinderpest sera, irrespective of the source (Table 314; Fig. 96). However, the titres of measles ID antigens and Tween-ether haemagglutinins were significantly reduced after adsorption at $4^{\circ}C$ for 24 hours with anti-rinderpest sera ($t = 15.588^{**}$; $P < 0.001$ and $t = 12.388^{**}$; $P < 0.001$ respectively) (Table 315)

RINDERPEST AND DISTEMPER

Anti-Rinderpest Sera: Antibodies reactive to distemper CF and ID antigens and cell culture virus were recognized in cattle, goat and rabbit anti-rinderpest sera (Table

314). The precipitation lines that developed in the ID tests merged completely with control precipitation lines that developed between the anti-distemper sera and distemper antigens (Fig. 96). Moreover, in adsorption tests, the titres of distemper ID tissue antigens were significantly depressed after incubation with anti-rinderpest sera ($t = 10.000^{**}$; $P < 0.001$)(Table 316).

When ox and rabbit anti-rinderpest sera were incubated for 24 hours at $4^{\circ}C$ with distemper ID antigens and the mixtures were diffused in agar gel against anti-distemper serum precipitation did not occur (Fig. 96).

The N antibody titres in rabbit sera were significantly higher than the CF titres ($t = 3.709^{**}$; $P < 0.01$). The CF titres were in turn significantly higher than the ID titres ($t = 4.286^{**}$; $P < 0.001$) (Table 314).

DISCUSSION

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VIRUS ISOLATION

Measles Virus: Measles virus has a reputation of easy isolation from infected human tissues in PMK cells (Matumoto, 1966). I likewise, had no difficulty in isolating the virus in PMK cells and we were also able to isolate it in Vero cells but not in other simian and human cell lines. My success using Vero cells can be attributed to the fact, first noted by Desmyter, Rawls, Melnick, Yow and Barret (1967), that Vero cells are unable to produce interferon.

Canine Distemper: Isolation of canine distemper virus from distemper-infected tissues was difficult. Virus was isolated from the lung tissues of only three out of nine cases of clinical distemper despite the fact that all the specimens contained specific CF and ID antigens and virus particles were detected on electron microscopic examination in eight of the nine specimens. Moreover, maintenance of the isolates in primary canine kidney cells was also difficult; two strains were lost on serial passage.

Published reports of isolation and propagation of the wild strains of canine distemper virus in cell cultures are meagre, and few investigators have furnished details or cited success rates. Rockborn (1958a) recovered canine distemper virus in PCK cells from the pooled serum of two dogs experimentally infected intranasally with tonsillar and nasal washings

from clinical cases of distemper. Vantsis (1959) prepared cultures from the lungs and kidneys of 12 naturally infected dogs and from the kidneys of seven experimentally infected ferrets and demonstrated the virus in all cultures. He also claimed to have isolated the virus in primary canine and ferret kidney cell cultures from "numerous" cases of distemper-infected dogs. This contrasts with the singular failure of Cabasso, Kiser and Stebbins (1959) to propagate wild strains of canine distemper virus in PCK and PFK cell cultures despite repeated efforts.

Bussell and Karzon (1965a) gave details of their experimental methods. Virus was isolated from only one out of five natural cases of dog distemper. The onset of the CPE in PCK cells was delayed up to 29 days and the involvement of the cell sheet was about 50 per cent at the end of the 5th or 6th week. Visual recognition of the CPE had to be confirmed by staining the cultures. Moreover, infectivity titres were poor. Our findings are in accord. Bussell and Karzon (1965a) also underscored their failure to isolate the virulent Snyder Hill strain of canine distemper virus by direct inoculation of infected spleen suspensions into primary ferret kidney (PFK) cells. Likewise, kidney cell cultures prepared from two ferrets infected with two other virulent strains of the virus failed to develop any CPE, although the ferrets developed the characteristic

temperature response and illness.

My failure to isolate canine distemper virus from most clinical specimens can be ascribed to several factors. The nine lung specimens were obtained from cases of clinical distemper illness of more than two weeks duration. Although the suspensions were rich in CF and ID antigens, the concentrations of the infective virus may have been insufficient to initiate detectable infection in the inoculated PCK cells. Moreover, interference due to non-infectious virus particles is a possibility. This phenomenon has been demonstrated in measles-infected cultures (Drevo and Mares, 1967).

The mammalian lung is rich in lympho-reticular tissue (Florey and Gowans, 1964), a favourable milieu for antibody production. It is conceivable that there was co-existence of antibody and virus in the lung specimens. The process of preparation of the tissue suspensions may have entailed neutralization of the virus particles liberated from the lung cells. It is noteworthy that Rockborn (1958a) used the acute-phase serum to infect the PCK cell cultures. We believe that the time of sampling of the infected tissues is important in the isolation of canine distemper virus in cell cultures; a fact not stressed in the published accounts.

The observation of Rockborn (1958a) and Bussell and Karzon (1965a) that the distemper CPE in PCK cells

affected only the epithelial but not the fibroblastic elements is perhaps significant. It is possible that the susceptibility of the canine renal cells to infection with the virus is influenced to a degree by the preponderance of the epithelial cells in the cell sheets.

The efficiency of virus replication may be impaired by the presence of interfering (Rubin, 1960) and the helper (Hanafusa, Hanafusa and Rubin, 1963) viruses. Canine kidney cultures are reported to contain latent viruses (Simonyi, 1963; Spertzel, Huxoll, McConnell, Bun and Yager, 1965). In a laboratory which did not handle canine distemper virus, Kasza (1966) isolated canine distemper virus from subcultured PCK cells. The CPE became pronounced after the 4th blind passage. A recent Japanese report (Domoto, 1969) elucidates the adverse effects on the infectivity titres of infective canine hepatitis virus by the so-called "adeno-associated satellite virus" latent in the PCK cells. Per se, the latter agent did not induce CPE, but multiplied symbiotically with the infectious canine hepatitis virus. The inhibitory or salutory effects of latent agents on the growth of canine distemper virus, therefore, merit further study.

Growth of animal viruses in cell cultures is known to be associated with the production of interferon (Isaacs and Lindenmann, 1957; Isaacs, 1963). In the present study, direct evidence for the presence of

interferon in PCK cells infected with the virulent isolates of canine distemper virus was not sought. However, the fact that the infectivity titres of the CTVM strain were significantly higher in MDCK cells pre-treated with actinomycin-D than in the untreated cells supports the presumption that the increased infectivity was associated with the inhibition of interferon synthesis. The loss of two virulent isolates of canine distemper virus on serial passage in PCK cells may be attributed in part to the admixture of harvested interferon with the seed virus used in the passage inoculations.

CYTOPATHOGENICITY

Measles: Measles virus strains were easily adapted to grow in human and simian primary cell cultures and cell lines. Serial passage of the virus entailed a progressive diminution in the time of onset of the CPE. In general, the spread of the CPE was rapid and multicentric and the involvement of the cell sheet maximal. Haemadsorption was the hallmark of measles CPE, but even without the aid of haemadsorption visual assessment of the CPE was always reliable. The onsets of the CPE and haemadsorption were inversely and significantly related to the dose of the inoculated virus and the age of the cells.

Canine Distemper: In our hands, the strains of canine

distemper virus had a narrow spectrum of infectivity. The CTVM and Rockborn strains infected the canine cell cultures only. Likewise, the Onderstepoort strain was propagated in fowl embryo cells only. Bussell and Karzon (1965a and 1965b) adapted distemper virus strains of different passage histories to human, simian, ferret and bovine cell cultures and postulated that the selection of appropriate mutants was the prerequisite for a strain to be continuously propagated with marked CPE in any given cell type. Our inability to adapt the virulent CTVM and the Rockborn strain of canine distemper virus to other mammalian cell cultures apparently stemmed from a single basic factor, namely the insufficient number of serial passages carried out.

Comparisons: One of the primary objectives of the present study was to attempt to compare the behaviour of the two viruses in common host cell systems. This was not achieved due to failure of attempts to adapt canine distemper virus to cell cultures in which measles virus grew with ease. However, comparisons are justifiable on the grounds that the features of cytopathogenicity of the two viruses were largely similar irrespective of the host cell types.

Effect of age of cells: The onsets of measles and canine distemper CPE were inversely and significantly related to the age of the cultured cells. With distemper the age of the cells was more decisive in PCK

than in MDCK cells, but no real strain differences existed. Infectivity studies were limited. Nevertheless, titres in either cell type were not apparently related to the age of the cells.

The negative correlation between the age of tissue culture cells and the CPE or virus titres has been the subject of several reports. Plowright and Ferris (1957) noted that the CPE of rinderpest virus in calf kidney cells was most striking in areas of the monolayers containing "younger" and dividing cells, and recommended the use of trypsin-dispersed cells rather than fully formed monolayers for obtaining higher virus yields and earlier detection of the CPE. Cheever and Wilmet (1942) observed that Herpes simplex virus did not multiply in cultures of fowl embryo fragments more than four days old. In human amnion cells infected with a strain of type 2 HEF-1 polio virus the inhibitory activity against polio virus type 1 was significantly higher in 19-35 days' old cultures than in 13 days' old or younger cultures (Ho and Enders, 1959). Similarly, Libikova and Smidova (1962) noted an inverse relationship between the age of the cultured HeLa cells at the time of infection and the CPE induced by the tick-borne encephalitis virus. The propensity of the younger cells to induce earlier CPE and higher yields of virus was attributed to the production of significantly lesser amounts of interferon than the older cells

(Carver and Marcus, 1967; Henslova and Libikova, 1966; Libikova, Rajcani and Henslova, 1969). Older cells not only produced more interferon but were also more sensitive to its action than the younger cells (Carver and Marcus, 1967). These investigators postulated that the amount of interferon synthesized in cells reflected an equilibrium between the cell-derived repressor and the virus-induced derepressor interaction. It was thought that in the absence of infection the cell contained sufficient number of repressor molecules to maintain control over interferon production. Following infection the virus-coded derepressor produced by the cell interacted with the repressor, the equilibrium determining the rate of interferon-mRNA transcription. The authors speculated that the efficacy of the transcription of interferon-mRNA was greater in the aged cells than in the younger cells. Levine, Becker, Boone and Eagle (1965) demonstrated that the rates of RNA, DNA and protein synthesis decreased considerably as cultures of human diploid cells attained confluency or high levels of contact inhibition. Contact inhibition seemed to be operative from about the 3rd day of the ageing process (Carver and Marcus, 1967). It is conceivable, that the overall reduction in macromolecular synthesis in aged, contact-inhibited cells results in a decrease in the production of repressors.

Measles virus is known to induce interferon

synthesis in different cell culture systems (DeMaeyer and Enders, 1961; Greser, 1961). The ability of canine distemper and rinderpest virus to induce interferon *in vitro* however, remains to be elucidated. In our studies of the Onderstepoort strain of canine distemper virus, a negative correlation was demonstrated not only between the age of fowl embryo cells and the time of induction of the CPE but also between the age of the embryos, the source of the cells, and the onsets of the CPE. These findings are consistent with the demonstration of significantly higher yields of interferon from older embryos than from younger embryos (Baron and Isaacs, 1961).

Incubation temperature effect: In general, cultures held at higher temperatures developed earlier CPE than the cultures incubated at lower temperatures. Statistically significant relationships between the incubation temperature and the onsets of the CPE however, emerged in only trials with the CTVM strain of distemper. Ensyme reactions are temperature-dependent and it is reasonable to assume that higher temperatures may have accentuated the metabolic activities of the cells leading to a quicker induction of cytopathic changes. However, the validity of this hypothesis has to be examined in the light of the observation that interferon production is also directly and significantly related to increases in the temperature of incubation (Waschke,

Lackovic and Borecky, 1969). In the present study, young cell cultures were used and the virus inocula were allowed to adsorb on to the cells for two hours at room temperature, parameters that are known to adversely affect the induction of interferon.

Effect of actinomycin-D: Results of several trials with the CTVM and the Rockborn strains of canine distemper virus revealed that the onsets of the CPE occured significantly earlier in cells pre-treated with actinomycin-D than in untreated cells. Likewise, FL and MDCK cells pre-treated with the drug and infected respectively with measles and canine distemper viruses, showed higher infectivity titres than the untreated cultures. Furthermore, the evolution of the infectivity in FL cultures inoculated with measles virus was related to the dose of the drug. Our data are in accord with the findings of Anderson and Atherton (1964) and Matumoto et al. (1965) with respect to measles virus replication in the presence of actinomycin-D. Actinomycin-D has been shown to inhibit DNA-dependent, DNA-primed, RNA synthesis in vitro (Goldberg and Rabinwitz, 1962). The fact that the replication of measles virus is unaffected by the presence of actinomycin-D suggests that measles virus is an RNA virus and that its replication is independent of DNA-primed, RNA synthesis of the host cell. This is in accord with the observations of Reich (1963) that

actinomycin-D at concentrations sufficient to suppress cellular RNA synthesis completely, permits replication of several RNA viruses. The effect of the drug on canine distemper and rinderpest viruses has not yet been investigated.

The salutary effects of actinomycin-D on measles virus replication were attributed to the suppression of interferon production in fowl embryo fibroblasts (Anderson and Atherton, 1964). Whereas untreated cells having an interferon titre of 1:12 produced fewer than 10 PFU/ml of the virus, cells pre-treated with actinomycin-D contained no demonstrable interferon and yielded $10^{2.7}$ PFU/ml of virus. The inhibition of interferon production by actinomycin-D in fowl embryo cell cultures inoculated with Newcastle disease virus has been demonstrated by Friedman (1964). However, quantitative data are lacking regarding the production of interferon by the "medipest" viruses in vitro.

Dose effect: The effect of dose of inoculum on the development of measles and distemper CPE was profound. Moreover, the onset of measles haemadsorption was significantly related to the dose of inoculated virus. Larger amounts of the virus probably permit the establishment of extensive areas of infection in the cell sheet resulting in faster, multicentric spread of infection.

Differences in the cytopathogenicity of measles and

distemper viruses were mainly related to their rates of growth in cell culture systems. Infectivity titres of distemper virus were low. Serial passage in canine cell cultures did not lead to any appreciable exhaltation of titres nor did reduction in the onset of the CPE occur. In addition, the effect of various animal sera on distemper CPE was pronounced; best results were obtained with foetal calf serum. Serum inhibitors active against influenza viruses and their antigens have been demonstrated and characterized (Cohen, Newland and Biddle, 1963; Choppin and Tamm, 1964). In their studies of propagation of canine distemper virus in canine lung macrophage cultures Appel and Jones (1967) obtained good yields of virus with media containing newborn lamb serum and rabbit serum but not with those containing bovine, equine, porcine, canine and guinea pig sera. Laboratories in countries endemic for rinderpest must take into consideration the possible occurence of antibodies reactive against canine distemper virus in calf sera.

CYTOMORPHOLOGY

Infection of mammalian cell cultures with measles and distemper viruses resulted in the development of lesions, the hallmarks of which were the formation of syncytia and stellate cells. The Onderstepoort strain of canine distemper virus, however, did not induce

syncytia in fowl embryo cell cultures, a well-documented phenomenon (Karzon and Bussell, 1959; Bussell and Karzon, 1962; Prydie, 1968). The number, distribution and morphological features of measles and distemper syncytia varied in different host cell systems. In general, the virulent strains induced larger and more numerous syncytia in primary cell cultures. The adsorption of simian erythrocytes was pathognomonic of measles syncytia.

Measles and distemper cultures also revealed the presence of cytoplasmic and nuclear inclusions. With canine distemper virus, the inclusion bodies were large, numerous and occured regularly in PCK cultures infected with the CTVM and Rockborn strains. Nuclear inclusions were scanty or difficult to recognize in MDCK cells infected with two strains. The Onderstepoort strain did not induce inclusion body formation.

All strains of measles virus induced cytoplasmic and nuclear inclusions in PMK cells. Their frequency of occurence decreased during serial passage in human and simian cell lines. The three adapted strains failed to produce the inclusions in the later passages in LLC-MK2, BSC-1 and Vero cells. These findings are in harmony with the observations of Black et al. (1959) and Norrby et al. (1964a) that the inclusion bodies were not demonstrable in human cell line and in LU106 cells respectively.

In contrast, the cytopathology of rinderpest, while similar to those of measles and canine distemper, is characterized by the constant development of syncytia, nuclear and cytoplasmic inclusions irrespective of the host cell system and irrespective of the virus strain (Plowright and Ferris, 1957, 1959a; Huygelen, 1960; Gilbert and Mornier, 1962a, 1962b; Tokuda et al., 1962; Plowright, 1963a; Liess and Plowright, 1963b; Liess, 1964; Johnson and Ritchie, 1969).

The onsets of syncytia, cytoplasmic and nuclear inclusions in measles and distemper cultures were inversely and significantly related to the dose of the inoculated virus, a finding in close agreement with the observations of Dawson (1964) on the cytopathogenicity of a bovine strain of parainfluenza III virus. In the present study, the onsets of nuclear inclusions in measles and distemper cultures were later than the onsets of syncytia and cytoplasmic inclusion bodies. Similar observations were made in cell-cultures infected with rinderpest (Plowright and Ferris, 1957; Gilbert and Monnier, 1962a) and "peste des petits ruminants" (Gilbert and Monnier, 1962b). Moreover, the nuclear inclusions in cultures infected with "peste des petits ruminants" were larger and more numerous when the cultures were held at 40° than at 37°C. In our studies of measles cytopathology, the cytoplasmic and nuclear inclusions were fewer and smaller in cultures held at

33° than at 37° and 39°C.

VIRAL ANTIGENS

Measles Haemagglutinins: Titres of haemagglutinins in the culture fluids were directly and significantly related to infectivity titres. Irrespective of the cell type or the strain of virus used, haemagglutinins were demonstrable only when the infectivity titres were $10^{4.9}$ TCD_{50} 1/ml or greater. Some of the early reports on measles haemagglutination mentioned low haemagglutinin titres in cell cultures (Mastyukova and Khait, 1960; Rosen, 1961; Rosanoff, 1961; Togo, 1964). The ratios of haemagglutinin titres to infectivity titres quoted by Peries and Chany (1962) and Norrby (1962a) are somewhat lower than ours. Their data, however, stemmed from restricted studies using only one type of cell culture.

Studies on the kinetics of haemagglutinin production in FL and PMK cells with the virulent Belfast strain and the Holly Hill adapted strain of measles virus revealed a parallel development of infectivity and HA titres. Neither the age of cells nor the temperature of incubation of cultures had a significant effect on the yields of haemagglutinins. These parameters were possibly not sufficiently sensitive to differentiate small differences in the yields because in most cell systems the production of haemagglutinins was low. The influence of the dose of virus and pre-treatment of

tissue culture cells with actinomycin-D was, however, significant. Enhanced titres resulting from actinomycin-D treatment of cells strengthen the accepted hypothesis that the production of haemagglutinins is virus-coded and is independent of the host cell DNA-primed mechanisms.

Cell-bound haemagglutinins were titrated by sonication or freezing and thawing. The HA titres of the crude suspensions were increased several fold by Tween-ether or sodium deoxycholate treatments, the higher efficiency of the latter procedure being well-documented (Laver, 1963; Norrby, 1966). The enhancement of HA titres resulting from the splitting of measles virus with Tween-ether or sodium deoxycholate was shown to be due to the conversion of the "large" haemagglutinins into the morphologically homogeneous "small" haemagglutinins (Waterson, 1965; Norrby, 1967). Electron microscopic evidence for this finding has been presented (Waterson et al., 1963; Norrby, 1964a).

Complement-Fixation Antigens: Like the production of haemagglutinins the production of CF antigens in measles-infected cultures was poor. In general, the parameters of the CF antigen production were similar to the parameters of the haemagglutinin production except that there was no clear-cut link between infectivity and CF antigen activity in cell cultures. Further, CF antigens were detected earlier than haemagglutinins, a

finding which is in agreement with that of Numazaki and Karzon (1966).

Data on chemical characterization of measles, rinderpest and canine distemper CF antigens are meagre. With rinderpest and canine distemper, most studies were carried out with tissue antigens. Rinderpest CF antigens appeared to be proteins (Boulanger, 1957a) and were thermostable (Nakamura, 1958). Data on the thermostabilities of measles and canine distemper CF antigens are not always in agreement. Girardi et al. (1958) reported reduction of measles CF titres when exposed for 30 minutes to 56°C. Maeyer and Enders (1961) and Norrby (1967), however, noted no adverse effects. With distemper, Laidlaw and Dunkin (1931) found that CF antigenic activity in tissue suspensions was greatly reduced by heating for one hour at 50°C; the activity was totally absent in suspensions heated to 60°C for 30 minutes. Bussell and Karzon (1965b) reported that whereas the CF antigens from infected fowl embryo cell cultures were thermolabile at 56°C for 30 minutes, the antigens derived from mammalian cell cultures resisted the treatment. In the present study, distemper and measles tissue and cell culture CF antigens were thermostable at 56° to 60°C for 20 to 30 minutes.

Immuno-Diffusion Antigens: The presence of ID antigens in measles and canine distemper cell cultures was hard to demonstrate; concentration procedures were necessary.

Distemper-infected tissues were, however, rich in ID antigens. Their distribution in the different tissues of infected dogs and ferrets was largely similar to that reported by Mansi (1958) and Fraser (1966). In rinderpest-infected cattle, Scott and Brown (1961) demonstrated specific ID antigens in 18 tissues, but none was so rich as the lymph nodes. It is interesting to note that the tissues rich in ID antigens were also rich in virus (Walker et al., 1946b; Scott, 1955a).

Like the CF antigens, measles and distemper ID antigens were thormostable and appeared to be proteins. Further, the CF and ID antigens of measles and distemper appeared to be closely related because concentrated tissue or cell culture preparations of the two viruses exhibited both activities, although differing in degree and the two types of antigens were precipitated by ammonium sulphate of similar concentrations. The development and distribution of ID and CF antigens in rinderpest-infected cattle was reported to be similar (Scott, 1967).

MEASLES HAEMAGGLUTINATION

Measles haemagglutination has certain inherent, but as yet, unexplained physico-chemical features which differentiate it from the haemagglutination of other viruses. Measles haemagglutination has a restricted cell spectrum; only simian erythrocytes are

agglutinated (Rosanoff, 1961; Matumoto, 1966). A Russian claim (Lozovskaya, 1961) that the ch-3 strain of measles virus propagated serially in monkey kidney, Hep-2 and fowl embryo cell cultures agglutinated human, monkey, sheep, fowl, dog and guinea pig erythrocytes is unconfirmed. The author gave no details of the serological specificity of the reaction. We examined the parameter in detail using Tween-ether haemagglutinins and RDE-treated and untreated suspensions of erythrocytes from different species of mammals and birds and carrying out the tests under different hydrogen-ion concentrations and temperatures of incubation. We did not detect receptors for measles haemagglutinins in the erythrocytes of the species examined. However, an observation of interest was that some samples of human 'O' blood group erythrocytes pre-treated with RDE were agglutinated to a low titre. The use of human 'O' red cell membrane suspensions in adsorption tests with the haemagglutinins may shed light on the specificity of the reation and warrant further study.

The sensitivity of erythrocytes from different simian species is reported to vary (Cutchins, 1962; Ruckle-Enders, 1962; Funahashi and Kitawaki, 1963). We observed a gradient of sensitivity; the highest titres were obtained with baboon erythrocytes and the lowest with rhesus and talapoin cells. Moreover in adsorption tests the sensitivity of erythrocyte suspensions was

significantly augmented by pre-treatment with neuraminidase (RDE) such that higher dilutions of the haemagglutinins agglutinated the treated erythrocytes. The reaction was dose, temperature and time-dependent. The slopes of the regressions of adsorption of haemagglutinins by enzyme-treated, baboon, rhesus and cynomologus erythrocytes were similar but the level of the regression line of adsorption by the baboon erythrocytes was significantly higher than those of the other two regressions suggesting that the baboon erythrocytes were the most sensitive to the enzyme treatment and to haemagglutination. Our findings were in accord with those of Tischer (1967), who reported increased HA titres with the enzyme-treated rhesus and cynomologus monkey erythrocytes and postulated that neuraminidase-treatment engendered reduction of the electric surface potentials on the erythrocyte receptors resulting in diminished uptake of haemagglutinins. On the other hand, Norrby (1962b) and Waterson (1965) reported that the erythrocyte receptors for measles haemagglutination were insensitive to the action of neuraminidase.

Another fundamental difference between haemagglutination by measles virus and haemagglutination by other influenza and parainfluenza viruses was that the interaction between erythrocytes and measles haemagglutinins was irreversible. In our hands efforts to dissociate the adsorbed haemagglutinins from the

erythrocytes by prolonged incubation at ambient temperatures were unsuccessful. Likewise, pretreatment of the erythrocytes or treatment of the erythrocyte-haemagglutinin complexes with neuraminidase resulted in failure to release the haemagglutinins. Several workers (Peries and Chany, 1960, 1962; Norrby, 1962b; DeMeio and Gower, 1961; Karzon, 1962; Waterson, 1965) have underscored their failures to obtain spontaneous elution of measles virus or haemagglutinins from the erythrocytes. Their findings are in contradistincion to the report of Schluederberg and Nakamura (1967) that the reaction between simian erythrocytes and the so-called salt-dependent measles haemagglutinins is reversible. This report is neither confirmed nor repudiated.

According to Peries and Chany (1962) and Bussell and Karzon (1967), a striking difference between the erythrocyte receptors for measles haemagglutination and those for other myxoviruses was that the receptors for measles haemagglutinins were destroyed by formaldehyde. However, in our studies, formalinized simian erythrocytes were agglutinated by measles Tween-ether haemagglutinins. Moreover, cell cultures infected with measles virus showed haemadsorption of formalinized simian erythrocytes. We found that the technical difficulties attendant upon the preparation of stable suspensions of formalinized erythrocytes were considerable. Fresh blood samples were more suitable than

stored samples because the latter showed a greater tendency for haemolysis. Furthermore, haemolysis occurred either during formalin-treatment or centrifugation of the treated cells or even later, on storage of washed cells. Haemolysis that occurred during formalin-treatment was temperature-dependent.

The HA titres of formalinized simian erythrocytes were inversely and significantly related to the concentration of the reagent. The regressions of formalin-inactivation of simian erythrocytes and the erythrocyte membranes were similar, but the regressions of inactivation of the haemagglutinins differed from those of the erythrocyte membrane suspensions.

Measles haemagglutinins were apparently lipoproteins (Peries and Chany, 1962; Norrby, 1962a, 1962b; Waterson, 1965). Their activities were reduced or sometimes even abolished by chloroform and ether-treatments (Norrby, 1962b). In our studies, the rates of interaction of measles haemagglutinins with simian erythrocytes were significantly and linearly related to the temperature of incubation. In adsorption tests, the rate of adsorption of the Behringwerke Tween-ether haemagglutinins by the erythrocytes at each temperature was linear and significant. An Arrhenius plot of the adsorption rates was, likewise, linear and significant suggesting that the haemagglutinin-erythrocyte reaction followed first-order kinetics.

Further evidence of the proteinaceous nature of the haemagglutinins stemmed from studies of formalin and trypsin-inactivations. The rates of inactivation of the haemagglutinins by trypsin at 37°C were linearly and significantly related to the concentrations of the enzyme used. An Arrhenius plot of the values obtained at various concentrations of trypsin revealed that the inactivation followed first-order kinetics. Moreover, trypsin-degradation of measles haemagglutinins was influenced by pH in a manner reminiscent of a protein degradation reaction. Inactivation was characteristically most rapid at the pH 8.4.

The thermal decay of the different preparations of measles haemagglutinins were typical first-order reactions. Statistically however, the Behringwerke Tween-ether preparation was more thermostable than the rest. Furthermore, the Behringwerke Tween-ether haemagglutinins were thermostable over a wide range of pH; significant linear inactivation occurred at the pH limits only. Hirst (1948) pointed out that the influenza haemagglutinins (PR 8 strain) were active over a wider pH range, namely, 2.4 to 10.3. This contrasts with the pH stability range of 5.9 to 9.8 quoted by Chu (1948) for vaccinia haemagglutinins. On the basis of our finding of the thermostabilities of measles haemagglutinins it is tempting to agree with Norrby's suggestion (Norrby, 1962b) that measles haemagglutinins

were perhaps more closely related physico-chemically to vaccinia haemagglutinins than to influenza haem-agglutinins. The fact that one component of the vaccinia haemagglutinin has been demonstrated to be sensitive to trypsin action (Younger and Rubinstein, 1959) and that vaccinia haemagglutination is not followed by spontaneous elution assumes significance in this context. Moreover, Norrby (1962b) succeeded in dissociating measles haemagglutinins from the agglutinated erythrocytes by means of anti-measles serum, a method described previously for vaccinia (Burnet and Stone, 1948).

Lyophilization reduced the activity of measles haemagglutinins. This finding is in agreement with that of Waterson et al. (1963) with respect to measles CF antigens but contradicts that of Maurice, Provost and Borredon (1969) that lyophilization destroyed the activity of measles Tween-ether haemagglutinins.

The results of our studies on the effects of oxidizing agents on measles haemagglutinins were in accord with those of Norrby (1962b). He put forward the view that measles haemagglutinin molecules probably did not contain reactive carbohydrate groups. On the other hand, Waterson et al. (1963) contended that the denaturation of the haemagglutinins by potassium periodate reflected the presence of glycoproteins in the molecule. The original report of Hoyle (1952) on the

mode of action of potassium periodate on influenza haemagglutinins merits consideration in this context. Hoyle (1952) demonstrated that at higher molar concentrations, potassium periodate attacked proteins preferentially. Hoyle and Davies (1961) and Hoyle (1964) also have elegantly shown that the haemagglutinins of influenza virus were exponentially destroyed by iodine through inactivation of amino-acids, particularly histidine. It is also known that iodine affects the lipids of influenza and vaccinia viruses (Hoyle, 1964). These findings are consistent with Norrby's observations (Norrby, 1962b) that lipid solvents and urea destroyed measles haemagglutinins and that the haemagglutinins were probably lipoproteins.

Chemical characterization of the receptors for measles haemagglutinins on the surface of monkey erythrocytes is incomplete. Our studies suggested that the receptors apparently differed chemically from the haemagglutinins. The capacity of the simian erythrocyte membranes to adsorb measles haemagglutinins was linearly reduced when exposed to 56°C for 30 minutes. Treatment with oxidizing agents caused no adverse effects on the reactivity of baboon erythrocytes to measles haemagglutinins. On the other hand, trypsin and formalin induced slow, exponential inactivation suggesting the presence of reactive protein groups.

INHIBITION OF MEASLES HAEMAGGLUTINATION

Serological specificity of the measles HI test was restricted to the members of the measles-rinderpest-distemper triad. In the HI tests no evidence of cross-relationship between measles and the other classified animal and human myxoviruses was detected. It is, however, noteworthy that two samples of sera from goats, convalescent of a natural attack of "peste des petits ruminants", contained antibodies reactive to measles haemagglutinins. The immunological link between this disease and rinderpest has been discussed (Mornet et al., 1956a and 1956b) but no evidence exists of a serological relationship between measles and the agent of "peste des petits ruminants".

The measles HI test offers several advantages in the detection of antibodies to "medipest" viruses. It is simple and easy to perform. The procedure required for standardization of the antigen is simple in comparison with that needed for CF and N tests. Haemagglutination end points are sharp and clear-cut. Equivocal reactions can be confirmed by reshaking the contents of tubes or plates and recording the readings after allowing the sedimentation of the non-agglutinated erythrocytes to occur (Rosanoff, 1961; Norrby, 1962a). An additional advantage of the test emerged in the studies of Togo (1964). Some specimens of human sera containing low titres of measles antibodies could not be

tested in the N test as the lower dilutions of sera caused non-specific, toxic, degenerative changes in the cell culture. However, there were demonstrable titres of antibodies in the HI test. A similar difficulty is likely to arise with heavily contaminated sera. A minor snag of the test was emphasized by Kunita et al. (1963). Lower dilutions of sera often caused sedimentation of the added erythrocytes leading to button formation, a false positive reaction. In our hands, dubious reactions of this type were obviated by pretreatment of sera with kaolin.

We believe that the choice of the antigen is of paramount importance in measles HI test, particularly in the detection of antibodies to rinderpest and canine distemper. Titres were consistently and significantly higher with Tween-ether and sodium deoxycholate antigens than with crude preparations of the haemagglutinins. Low titres of antibodies were often missed with the latter antigens. To obtain reproducible results we recommend the incubation of the serum dilutions with 4 units of Tween-ether haemagglutinins overnight at $4^{\circ}C$ followed by the addition of 0.5 per cent suspension of baboon erythrocytes.

The measles HI test suffers from two minor shortcomings, neither of which, however, impairs the simplicity of the procedure. First, it is essential to remove the non-specific agglutinins from animal sera.

In our studies, sera of all species tested contained agglutinins for monkey erythrocytes. Adsorption of agglutinins was effective with packed or 50 per cent suspensions of erythrocytes or suspensions of erythrocyte membranes. Secondly, non-specific inhibition of measles haemagglutinins commonly occurred with cat, dog, ferret and rat sera. Literature on virus haemagglutination is replete with accounts of chemical pretreatment of sera for the removal of non-specific inhibitors. Procedures involving the use of reagents such as trypsin, potassium periodate, pseudomonas filtrates, acetone, protamine sulphate and the heparin-manganous chloride mixture are tedious, time-consuming and are unsuitable for large scale serological studies. In our hands, treatment with kaolin (Spence, 1960) was simple and effective. Mann, Rossen, Lehrich and Kasel (1967) reported the adsorption of immunoglobulins from sera following treatment with kaolin and recommended the treatment with a heparin-manganous chloride mixture as an alternate procedure. In our studies with several specimens of anti-measles sera of different sources treatment with kaolin for periods up to 24 hours at different temperatures did not entail significant reduction of specific antibody titres. Prolonged incubation with kaolin, however, resulted in a significant, linear reduction of the titres.

NEUTRALIZATION TEST

Studies on the thermal decay of measles and canine distemper viruses revealed that the viruses were relatively labile and that their responses to ambient temperature were of the same order. Moreover, the half-life values of five and three hours respectively for measles and canine distemper viruses were in the same range as the half-life value of 2.75 hours obtained for rinderpest virus (Plowright and Ferris, 1961). The decline in the titres of measles and distemper viruses at each temperature was linear and significant and followed first-order kinetics.

An examination of parameters such as the incubation temperature, time of incubation and virus dose with respect to each virus revealed similar linear regressions. With the lengthening of the incubation time, there were linear, significant increases in the neutralising antibody titres. Higher titres resulting from tests conducted at 37°C were assumed to be due to competition of non-infectious virus particles with the viable virus. The neutralization reaction was optimal at 25°C with respect to both measles and distemper systems. At this temperature the relationships between the dose of virus and the antibody titres were linear and significant such that an increase of 1 $\log_{10}$ unit of the virus depressed the antibody titre by 1 to 1.3 $\log_{10}$ units.

COMPLEMENT-FIXATION TESTS

Three parameters were examined and analysed statistically. The effects of incubation temperature and the concentration of complement on measles and distemper of reactions were pronounced and followed similar patterns. Titres at $4^{\circ}C$ were significantly higher than the titres at $37^{\circ}C$. Moreover there was a significant, inverse, linear relationship between the number of units of complement and the titres at $4^{\circ}C$. With both virus systems reproducible results were obtained when 4 units of the antigen were added to the dilutions of the test sera and the mixtures held at $4^{\circ}C$ overnight in the presence of 2 full units of complement.

IMMUNO-DIFFUSION TESTS

Measles and distemper immuno-diffusion reactions were typically temperature- and dose-dependent. The rate and magnitude of the reactions were directly and significantly related to the antigen or antibody concentrations. However, the rate of distemper immuno-diffusion reaction was faster than that of measles ID reaction ($F = 28.24^{**}$; d.f. 1, 19) (Fig. 98).

MEASLES ANTIBODIES

Natural Hosts: Serological studies of human and simian sera revealed direct, linear and significant relationships between the HI, CF and N antibodies. The levels

differed, the N antibody titres being always the highest, HI titres were higher than the CF titres. The data were in accord with the findings of other investigators (Norrby et al., 1963b; Enders-Ruckle, 1965; Krugman et al., 1965) and probably reflected the relative sensitivities of the three tests.

Most infant sera contained maternally-derived measles HI antibodies. The number of samples tested being few, no clear-cut relationship was demonstrable between the age of infants and the antibody titres. However, our findings were in agreement with the well-documented observations of the occurence of measles antibodies in 90 per cent of babies at birth (Enders-Ruckle, 1964).

Experimental Hosts: Measles virus did not induce a clinical state in rabbits, cattle and sheep. Nevertheless, these animals responded to the insult by producing different types of antibodies reactive to measles antigens. Seroconversion was 100 per cent irrespective of the route of inoculation.

The primary HI antibody responses of rabbits, cattle and sheep to inoculation with live measles virus were directly and significantly related to the dose of virus. Higher doses induced antibody production whereas lower doses did not. Moreover, antibody levels at 3-4 weeks post-inoculation were significantly related to the dose of virus, a finding that was reminiscent of that

reported for cattle inoculated with the highly attenuated cell culture rinderpest virus (Plowright and Ferris, 1959b). When inoculated with a dose of $10^{4.5}$ TCD_{50} virus, neutralising antibodies were detected in cattle on the 6th day. With failing doses, however, the onset of the antibody response ranged from the 7th to 17th day (Johnson, 1962c) or from 2 to 3 weeks (Plowright and Ferris, 1959b). Likewise, some Nigerian cattle reacting to "minimal" doses of caprinized virus did not develop antibodies until three months (Johnson, 1962b).

The onsets of HI antibodies and the rates of seroconversion were similar in rabbits inoculated with either live measles virus or inactivated haemagglutinins or Behringwerke Tween-ether haemagglutinins. All rabbits at 2-3 weeks post-inoculation had antibodies which persisted up to 10 months.

The primary HI antibody responses of cattle and sheep to live measles virus were similar to those of the rabbits both in onset and the rate of seroconversion but the levels differed. In cattle, antibodies persisted up to 11.5 months.

Colostral and colostrally-derived antibodies: Cows injected with measles virus or antigen concentrated measles antibodies in their colostrums; a finding in accord with that of Graves (1963) who measured colostral antibody titres in 10 heifers vaccinated with foot-and-mouth disease virus and found that 8 had higher titres

in their colostrums than in their sera. In our study, the colostral antibodies declined quickly, the rate of disappearance being linear and significant in three out of four cows.

The sera of mothers and their babies had at birth similar levels of measles antibodies (Ruckle and Rogers, 1957; Bech, 1961). On the other hand, sera of calves at birth, born of measles-sensitized cows, did not contain antibodies in their sera. In other words, there was no evidence of *in utero* transfer of antibodies. Haemagglutination-inhibition antibody titres developed six to eight hours following sucking, the onsets being collectively linear and significant. The time of onset of the antibodies in the sera of the calves in our study was longer than the figures quoted by Graves (1963) and Singh *et al*. (1967) in respect of foot-and-mouth disease and rinderpest neutralizing antibody titres in their experimental calves.

The mean half-life period of colostrally-derived measles HI antibodies in our study was 16 days which was in accord with the findings of Graves (1963) with respect to colostrally-derived foot-and-mouth disease antibodies whereas the mean half-life period of colostrally-derived rinderpest antibodies was reported to be about 30 days (Brown 1958b; Singh *et al*., 1967).

Antibody types: Neutralising, haemadsorption-inhibition and CF antibodies were also readily

demonstrable in the sera of rabbits sensitized with one dose of live measles virus or haemagglutinins. Haem-adsorption-inhibition antibodies were poor in cattle sera and undetectable in sheep and guinea pig sera. Immuno-diffusion antibodies were, likewise, undetectable in the sera of cattle, sheep and guinea pigs. These anti-bodies were regularly detected in the sera of rabbits inoculated with two doses of live virus or haem-agglutinins.

The onset of the CF antibodies in rabbit sera unlike that of the HI antibodies was generally delayed. CF antibody titres were lower than the HI antibody titres in rabbit and guinea pig sera. Studies in rabbits showed that they persisted for 6-8 weeks only. Irrespective of the source of serum, N antibody titres were always the highest.

DISTEMPER ANTIBODIES

Natural Host: We detected distemper CF antibodies in a high percentage of dogs of various ages and backgrounds but we were unable to relate the ages and the antibody titres. Rockborn (1958b, 1958c) similarly reported that many unvaccinated dogs had neutralising antibodies to distemper and, in contrast to our findings, he noted a positive relationship between the ages and the anti-body titres, a finding he attributed to the dogs having undergone subclinical infection. The discrepancy can

be explained by the differing natures of the two types of antibodies; for example, in measles infections in man CF antibodies decline more rapidly than N antibodies (Norrby, 1967).

Dogs that were ill from alleged distemper for more than 2-3 weeks had significantly higher CF antibodies than those that were ill for 7-14 days. Our data were in agreement with those of Mansi (1955) and Gorham (1960) who reported that in clinical distemper, CF antibody titres generally developed 3 to 4 weeks after infection.

Our serological examination of dogs convalescent of "clinical" distemper, revealed a close correlation between the CF and N antibody titres. As with human and simian measles sera, the N antibody titres in distemper sere were significantly higher than the CF antibody titres.

Our observations on maternally-derived antibodies to distemper in pups agreed with those of Gillespie (1956), Ott et al. (1957) and Gillespie et al. (1958) who showed that the passage of maternal antibodies was mainly through colostrum.

Experimental Hosts: Rabbits and cattle were clinically refractory to inoculation with live distemper virus. They, however, responded serologically to distemper antigens by producing CF and neutralising antibodies. As with measles, the response of rabbits and cattle to

distemper live or inactivated virus was significantly dose-dependent. The CF and N titres induced by the live virus were significantly higher than those induced by the inactivated virus. The N antibody titres were always higher than the CF titres. Only rabbits produced ID antibodies but the titres were poor.

ANTIBODY CURVES

On the basis of the immune responses of the hosts the "medipest" viruses belong to the second group of diseases in Francis's classification namely, diseases in which the infection is transient and the immunity is sustained. Beveridge (1952, 1963) characterized the group as the one having immunity of a high order. The long persistence of antibodies in measles, rinderpest and canine distemper is attributed to the wide dissemination of the viruses in the tissues and to their long replication in lymphoid tissues which provides a potent antigenic stimulus.

We studied the sequential development of the different types of antibody in rabbit sera. Our findings were broadly similar to those reported for human measles, bovine rinderpest and canine distemper.

In human measles antibodies appeared in blood one to three days after the rash and peak titres were reached 7 to 21 days later (Ruckle and Rogers, 1957; Bech, 1959). The onset of neutralising antibodies in cattle

depended upon dosage, virulence of the strain and host variation. Cattle inoculated with large doses of the virulent RBOK strain showed antibodies on the 5th or 6th day (Scott and Brown, 1958; Plowright and Ferris, 1962b) whereas those given the RGK/1 strain developed the titres on the 4th fever day (Plowright and Leiss, cited by Plowright, 1968). Peak titres were attained two to four weeks after the onset of illness (MacOwan, 1956; Plowright and Ferris, 1962b, 1962c; Johnson, 1962b, 1962c). Few detailed studies have been carried out on the development of antibodies in distemper. Gillespie et al. (1958) inoculated 420 pups aged 1 to 16 weeks with virulent virus. The earliest evidence of antibody was on the 6th day. Significant levels were sometimes detected on the 10th day and maximum titres were reached in 30 days. Similar data were furnished earlier by Baker et al. (1954), Ott et al. (1955) and Rockborn (1957a, 1957b) in dog and mink distemper.

Most studies in measles have revealed that the CF antibodies appeared in the primary response later than the HI and N antibodies (Stokes et al., 1961a; Krugman et al., 1965). Comparative studies on the persistence and degradation of measles HI antibodies after natural exposure or vaccination have revealed a correlation between the HI and N antibodies. Both were detectable for long periods of time than the CF antibodies (Krugman et al., 1965). A negative HI or N antibody

test was, therefore, a more sensitive index of susceptibility than a negative CF test.

Very little documented evidence exists in respect of the period of persistence of rinderpest N or CF antibodies. Significant titres were detected in cattle on the Pemba Island 13 years after immunization with caprinized virus (Brown and Raschid, 1958). The decline in neutralising antibody titres stimulated by low-passaged cell culture virus was more rapid between two weeks and six months after inoculation than in the following three or four years. High-passaged cell culture strains, on the other hand, produced antibodies that showed a slower reduction after peak titres at four weeks (Plowright, 1962c).

Limited data are available on direct comparisons of the N and CF antibodies. It is generally agreed that as with measles, the N antibody was a more reliable index of the immune status of the animal than the CF antibody (Walker et al., 1946; Ishii et al., 1953). Complement-fixing antibodies did not appear in all cattle sera which had recent contact with rinderpest virus. They were not detected in cattle inoculated with toluol-inactivated vaccine or with massive doses of the virus (Nakamura and Wagatuma, 1940). If they appeared in cattle sera it was usually about seven days after infection (Scott, 1967). The kinetics of the development of CF and N antibodies in rinderpest were similar

(Scott, 1967). Virus and both types of antibody were isolated from the same blood sample seven to nine days after the onset of fever which synchronized with the decline phase of viraemia (Scott, 1967). Titres of CF antibodies reached maximum in two or three weeks but their duration was variable (Cooper, 1946; Nakamura, 1958; Moulton and Stone, 1961). The presence of CF antibody indicated recent infection. As in measles its absence did not reflect a state of susceptibility to infection. Their detection is of little value in epizootiological surveys or in studies on the persistence of immunity following vaccination (Plowright, 1968).

Few studies have been carried out on the persistence of CF and N antibodies in distemper. Mansi (1965) reported that the CF antibodies were not demonstrable 15 weeks after natural exposure. Karzon et al.(1961) noted that in dogs inoculated with fowl embryo cell culture vaccine CF antibodies declined after 3-4 weeks and were not detectable after four months. It is generally agreed that the presence of CF antibodies is indicative of recent infection (Gorham, 1966).

In pups inoculated with the virulent Snyder Hill strain of canine distemper virus Gillespie et al. (1958) observed that the N antibodies persisted for at least two years. Keeble (1962) reported that the decline of N antibodies occurred at a rate of 0.5 $\log_{10}$ units

every six or seven months.

In multiple-vaccinated dogs the antibodies were demonstrable for four to six years (Piercy, 1961). In the Wellcome Research Laboratories, Beckenham, Prydie (1966) held 64 dogs, inoculated with the Onderstepoort strain of canine distemper virus, in isolation for two to six years. Following revaccination, 89 per cent had antibodies sufficiently high to neutralize the cell culture vaccine virus. Six of the seven dogs held longer than six years had levels of antibodies that were sufficient to neutralize similar concentrations of the vaccine virus.

CROSS-RELATIONSHIPS

Measles and Distemper: Naturally occurring strains of measles and distemper viruses are strictly zootropic. No evidence exists that the strains induce a clinical state in species other than their natural hosts. However, mouse-adapted strains produced identical syndromes and pathological lesions in sucking mice (Imagawa, 1965). Moreover, the clinical signs and histological changes induced by mouse-adapted strains of measles and canine distemper were indistinquishable from those produced by the mouse-adapted strains of rinderpest virus (Imagawa, 1969).

Claims in the literature of the alleged proliferation of measles virus in dogs (Bardach et al.,

1947; Rjazantseva, 1956; Goret, 1961) and of human pulmonary infections with canine distemper virus (Nicolle, 1931; Adams, 1941) were unsupported by virological data. Nevertheless, most human sera contained antibodies to canine distemper antigens (Karzon, 1955; Carlstrom, 1956, 1957; Bech, 1960; Black and Rosen, 1962). The converse namely the occurrence of measles antibodies in canine and ferret sera was reported by Arakawa et al. (1959) and Warren (1960) and confirmed by Imagawa et al. (1960) and Waterson et al. (1963). Contrary findings were published by Cabasso et al. (1959) and Roberts (1965). The discrepancy was attributed to variables such as differences in experimental methods, virus strains, animal hosts or cell culture systems, the size of inocula, the sensitivity of the antigens.

Dogs ill with distemper or with a distemper history developed antibodies in their sera to measles Tween-ether haemagglutinins and CF antigens. In some instances, neutralising antibodies to distemper virus were also demonstrated. On the other hand, even multiple inoculations of rabbits and cattle with live or inactivated distemper virus did not induce the development of antibodies reactive to measles haemagglutinins. Antibodies to measles virus and CF antigens were produced, however, the titres being related to the number of doses inoculated. In general, live virus induced higher titres than inactivated virus, and the

type of serological test employed (Imagawa, 1968). We examined the serological aspects of the relationships between measles and canine distemper using sera from natural and experimental hosts in comparable tests and our findings were in support of the one-way cross postulated by Roberts (1965), measles being the dominant partner.

We demonstrated antibodies reactive to distemper virus and CF antigens in most human and simian sera but the titres in human sera were generally poor or barely detectable. We noted a close correlation between the levels of antibodies to measles and distemper antigens in human and simian sera, a finding in accord with those of Bech (1960) and Black and Rosen (1962). They also demonstrated a significant, direct relationship between the titres of antibodies to distemper virus in human sera and the age of the subjects. Such a relationship was not evident in our studies which were restricted.

The origin of antibodies in man and monkeys reactive to distemper antigens is obscure. It is generally accepted that in man the stimulus for the production of antibodies to distemper is exposure to measles virus. The results of the extensive serological surveys of human populations in Greenland and Tahiti by Bech (1960) and Black and Rosen (1962) respectively supported this hypothesis. Canine distemper was unknown in Greenland and there was no canine population in Tahiti. Anti-

bodies reactive both to measles and distemper antigens developed in the sera of human patients, the homologous titres being the higher. Re-examination of the populations years later revealed that the differences in the levels of antibodies to measles and distemper were greatly reduced; in some instances the titres were identical. The phenomenon was attributed to the slower rate of decay of distemper antibodies.

In the present investigations, rabbits inoculated with measles virus or haemagglutinins developed antibodies reactive both to measles and distemper antigens. Similarly, calves and sheep inoculated with one or two doses of live measles virus produced neutralising antibodies to distemper but the titres were poor. However, no reaction was demonstrable when anti-measles sera from natural and experimental hosts were diffused in agar gel against distemper cell culture or tissue ID antigens. In adsorption tests, the titres of measles ID antibodies were largely unaffected when incubated with distemper ID antigens. Likewise, measles ID antigens showed no reduction in titres when adsorbed with anti-distemper sera.

A link between measles and canine distemper through their capsid antigens was evident in our studies of parallel CF tests. On the other hand, we failed to establish a link between the two viruses in agar gel precipitation reactions. There are two possible

explanations; one, the antigens reactive in complement-fixation and precipitation tests differ in character, or secondly, the antigens are the same and the difference is merely quantitative. Our findings, however, revealed that ID preparations were always rich in CF antigens and the thermostabilities of the two antigen preparations were similar. We therefore believe that the second explanation is the more likely because our ID preparations were either crude or partially purified. Further work is necessary to clarify the relationship, particularly the use of ID antigens purified on the lines described for measles CF antigens by Numazaki and Karzon (1966). However, irrespective of the source of antibodies no relationship was demonstrable between anti-distemper sera and measles haemagglutinins or concentrated cell-culture virus in agar-gel.

Anamnestic responses: Rabbits and calves primed with live measles virus or haemagglutinins responded anamnestically to reinoculation with live distemper virus producing measles HI and measles CF and distemper CF antibodies. The early onset and the rapid linear increase of titres suggested that the distemper CF antibody response was also anamnestic. Heat-inactivated distemper virus did not evoke the anamnestic responses; possibly because there was denaturation of the antigens.

Similarly, rabbits and calves exposed to live distemper virus developed significant anamnestic

titres of measles antibodies. Distemper N and CF antibody titres were not affected, a finding attributed to the distemper antibodies neutralising the components in measles haemagglutinins antigenically related to distemper envelope and capsid antigens.

Our findings of heterotypic antibody responses of sensitized dogs to measles and distemper antigens were in accord with those of Gillespie et al. (1960), Warren et al. (1960) and Moura and Warren (1961). However, our findings of homotypic and heterotypic anamnestic responses of sensitized calves differed from those of Delay et al. (1965). In their studies, cattle inoculated with live measles virus produced antibodies neither to measles nor distemper antigens, and cattle exposed to distemper virus produced distemper antibodies only.

We believe that our serological findings in respect of the natural and experimental hosts of measles and canine distemper justified the conclusion that the two viruses are distinctly related through their nucleocapsid antigens. A relationship through the envelope antigens possibly exists because sera of natural and experimental hosts showed neutralising antibodies to heterologous viruses. Moreover, heterologous challenge inoculations entailed anamnestic increases of the N antibody titres. Antibodies to measles haemagglutinins were demonstrable in some dog sera. We assume that the

occurrence of measles HI antibodies in dogs reflects the capacity of their tissues to permit replication of canine distemper virus in a manner leading to the production of antigenic determinants common to measles and distemper envelope antigens. This capacity which seems restricted to a few members of the species may be a genetic trait. Studies of organ cultures from different breeds of dogs infected with canine distemper virus may provide an answer to this intriguing phenomenon.

Measles and Rinderpest: Cattle, goat and rabbit anti-rinderpest sera contained antibodies that neutralized measles virus and were reactive to measles haem-agglutinins and CF antigens. The N titres were the highest and the CF titres the lowest. Detailed studies with sera of cattle, goats and pigs with a rinderpest history provided unequivocal proof that the measles HI test was efficient in the recognition of rinderpest antibodies; the antibodies were detected in a high percentage of these animals. We believe that measles and rinderpest are distinctly related through their envelope and capsid antigens. Morphologically the two viruses are similar (Waterson et al., 1961; Plowright et al., 1962). Reports of the demonstration of haem-agglutinins in rinderpest-infected cell cultures (Leiss, 1964; Provost and Borredon, 1968) were not supported by specific inhibition tests. Likewise, the report

(Anon, 1966) that pretreatment of patas monkey erythrocytes with rinderpest cell culture virus entailed reduction of reactivity with measles haemagglutinins awaits confirmation. However, the finding that the outer membranes of the two viruses are fringed and that the haemagglutinins of measles virion are located in the "spikes" (Waterson, 1965) justifies the presumption that the existence of a haemagglutinin in the rinderpest virion is a theoretical possibility (Scott, 1964). However, we could not demonstrate a relationship between measles and rinderpest in the agar-gel precipitation reactions possibly because of the crude preparations of measles cell culture antigens used in the test. The preparations contained CF antigens which fixed complement in the presence of rinderpest sera.

The sensitivity of the measles HI test in the recognition of rinderpest antibodies was evident in our studies of the sequential development of antibodies in cattle infected with the virulent bovine Hissar strain of rinderpest virus. In animals that died of the infection and in those that were clinically ill and destroyed <u>in extremis</u> antibodies reactive to measles haemagglutinins were detected on 6th day post-infection; simultaneously rinderpest virus was recovered from the blood and specific ID antigens were demonstrated in the lymph nodes. In his studies of the kinetics of the development of antibodies to rinderpest, Scott (1967)

demonstrated both virus and CF and N antibodies in the same blood samples seven to nine days after the onset of fever which synchronized with the decline phase of viraemia and the CF and precipitation curves in the lymph nodes. Bogel et al. (1964) demonstrated antibodies reactive to measles Tween-ether haemagglutinins in the sera of cattle nine days after infection with virulent and attenuated strains of rinderpest virus. In convalescent sera maximum titres were reached in two to three weeks post-inoculation. Our findings were in accord.

In a multiple vaccinated herd, antibodies reactive to measles Tween-ether haemagglutinins were detected in most animals; the titres persisted without significant reduction for 435 days. We believe that the long persistence of the HI antibodies in the cattle was not the result of multiple vaccinations because analyses of the primary and anamnestic responses revealed no relationships between the number of vaccinations and the titres of the antibodies. Moreover, in a group of calves inoculated with one dose of the cell culture vaccine the HI antibodies persisted without significant reduction of titres for 365 days. The findings were in accord with those of Bogel et al. (1964) and Rowe et al. (1967).

The HI antibody responses of goats were similar to those of cattle in onset and the rise in titres. On

the other hand, the titres fell more rapidly.

In West African Dwarf pigs the HI antibody response was not related to the severity of the clinical responses. The incidence of inapparent infections among domestic pigs naturally exposed is not known but the incidence of subclinical and non-clinical infections in experimentally infected pigs of European and North American breeds is high (Nakamura et al., 1957; Scott et al., 1959; Barber and Heuschele, 1964). The measles HI test, in our hands, detected specific antibody in pigs one week after exposure whereas neutralising antibodies, while present at 31 and 38 days after exposure, were not present at 10 days (Delay and Barber, 1962). The measles HI test may be of value as a screening procedure in serological surveys of rinderpest in pig population in areas where the disease is enzootic but further work is necessary to determine the persistence of HI antibodies in pigs.

Using the measles HI test we also detected colostrally derived antibodies to rinderpest in the sera of 8 of 27 calves, the progeny of vaccinated cows. The half-life of the colostrally-derived antibodies was 33 days, figure similar to that determined by Brown (1958b) and Singh et al. (1967). Calves with colostrally-derived antibodies did not respond to inoculation with cell culture vaccine; a finding reminiscent of that recently reported by French workers (Provost, Borredon and Maurice, 1969). The response of

calves to caprinized virus was, however, of a higher order both in the rate of development of the antibodies and in the levels.

Rinderpest and Canine Distemper: Antibodies reactive to distemper virus, CF and ID antigens were demonstrated in rinderpest sera. A clear-cut link between the two viruses was also evident in direct agar-gel precipitation tests and in adsorption tests. Lines of identity with those produced by anti-distemper sera emerged in agar-gel only with hyperimmune rinderpest serum, irrespective of the source. Our results were in accord with those of White et al. (1961) and Fraser (1966) but we did not note any spur formation with rabbit anti-rinderpest as reported by White et al. (1961).

CONCLUSIONS

Our studies of the serological relationships between the "medipest" viruses confirmed and extended established findings. The viruses are distinctly related through their envelope and capsid antigens. The nature and degree of the kinships are, however, controversial. Much of the confusion has stemmed from the failure to recognize that measles and distemper viruees behaved as non-replicating antigens in alien hosts.

In the natural hosts-virus systems no differences were detectable in the homologous and heterologous antibody responses (Tables 317 and 319). In primates,

measles was the most efficient antigen (Table 318). In cattle, the most efficient antigens were rinderpest and measles. In dogs, the most efficient antigens were distemper and measles. In hyperimmunized rabbits and cattle, rinderpest was the most efficient antigen for the production of heterologous antibodies in that it stimulated the development of antibodies reactive to distemper ID antigens, whereas measles did not (Table 320).

Challenge inoculation of the sensitized hosts with heterologous virus resulted in significant anamnestic responses characterized by the production of antibodies reactive to homologous and heterologous viruses, a finding which has practical implications. Heterotypic vaccination against the "medipest" viruses is not only feasible but even preferable in certain circumstances, for instance, measles vaccination of pups having colostrally-derived distemper antibodies. Measles immunizes against rinderpest and canine distemper. Distemper protects against rinderpest. There is also circumstantial evidence that rinderpest and distemper can prevent measles.

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